RECESSIVE PLANT VIRAL RESISTANCE RESULTS FROM MUTATIONS IN TRANSLATION INITIATION FACTOR eIF4E

[0001]   This application claims the benefit of U.S. Provisional Patent Application Serial No. 60/434,220, filed December 17, 2002, which is hereby incorporated by reference in its entirety.

[0002]   The present invention was made with funding received from the United States Department of Agriculture under USDA NRICGP Plant Genome Award No. 94-37300-0333 and USDA IFAFS Award No. 2001-52100-11334. The U.S. Government may have certain rights in this invention.

FIELD OF THE INVENTION

[0003]   The present invention relates to the methods of imparting virus resistance to plants by silencing a gene encoding a translation initiation factor eIF4E and/or by overexpressing in a plant a nucleic acid molecule encoding a heterologous translation initiation factor eIF4E.

BACKGROUND OF THE INVENTION

[0004]   Plant virus diseases can damage leaves, stems, roots, fruits, seed, or flowers, and are responsible for a considerable percentage of economic loss due to reduced crop yield and quality (Agrios, "Chapter 14: Plant Diseases Caused By Viruses," in *Plant Pathology*, 3rd Ed., San Diego, CA: Academic Press, p. 655 (1988)).

[0005]   Control of plant virus diseases took a major step forward when it was shown that the tobacco mosaic virus ("TMV") coat protein ("CP") gene that was expressed in transgenic tobacco conferred resistance to TMV (Powell-Abel et al., "Delay of Disease Development in Transgenic Plants that Express the Tobacco Mosaic Virus Coat Protein Gene," *Science* 232:738-43 (1986)). The concept of pathogen-derived resistance ("PDR"), which states that pathogen genes that are expressed in transgenic plants will confer resistance to infection by the homologous or related pathogens (Sanford et al., "The Concept of Parasite-Derived Resistance - Deriving Resistance Genes from the Parasite's Own

Genome," *J. Theor. Biol.*, 113:395-405 (1985)) was introduced at about the same time. Since then, numerous reports have confirmed that PDR is a useful strategy for developing transgenic plants that are resistant to many different viruses (Lomonossoff, G.P., "Pathogen-Derived Resistance to Plant Viruses," *Ann. Rev.*

5   *Phytopathol.*, 33:323-43 (1995)). However, additional modes of protecting plants against virus disease are needed.

[0006]   Recessive disease resistance genes are widely deployed in agriculture and are common in nature. However, not much is understood about the identity of naturally occurring recessive disease resistance genes in plants.

10   Plant disease resistance conferred by recessive genetic factors has received limited attention relative to dominant R genes, despite their durability and prevalence in nature. Thus, there is a need to identify, isolated, and clone plant host genes whose gene product is essential for pathogenesis. This knowledge would be useful in that it would enable one to engineer disease resistance the various gene

15   silencing methods available in the art.

[0007]   Potyviruses comprise approximately 30% of all known plant viruses and as a group are very destructive in agriculture (Ward et al., "Taxonomy of Potyviruses: Current Problems and Some Solutions," *Intervirology* 32:269-296 (1991)). The family Potyviridae is characterized by a monopartite single-stranded

20   positive sense RNA genome with a covalently-bound viral-encoded protein (VPg) attached at the 5' terminus and a 3' poly-A tract (Riechmann et al., "Highlights and Prospects of Potyvirus Molecular Biology," *J. Gen. Virol.* 73:1-16 (1992)). The genome is approximately 10 kb in length and is translated as a polyprotein which is subsequently cleaved into smaller polypeptides by viral-encoded

25   proteases. Based on similarities in genome structure, including conserved order and function among homologous replication proteins, potyviruses have been assigned to the proposed picorna-like superfamily of viruses, which includes many important human and animal pathogens, such as poliovirus and foot-and-mouth disease virus (Goldbach et al., "Genetic Organization, Evolution and

30   Expression of Plant Viral RNA Genomes," In Fraser , ed., *Recoginition and Response in Plant-Virus Interactions*, Heidelberg:Springer-Verlag, pp. 147-162 (1990); and Riechmann et al., "Highlights and Prospects of Potyvirus Molecular Biology," *J. Gen. Virol.* 73:1-16 (1992)).

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[0008]   Potyvirus infection requires the interaction of host factors with viral proteins and RNA for replication and systemic spread (Carrington et al., "Cell-to-Cell and Long-Distance Transport of Viruses in Plants," *Plant Cell* 8:1669-1681 (1996)). Although much is known regarding the functions of the individual potyvirus proteins and RNA structures in viral replication and movement (reviewed in Revers et al., "New Advances in Understanding the Molecular Biology of Plant/Potyvirus Interactions," *Mol. Plant Microbe Interact.* 12:367-376 (1999); Riechmann et al., "Highlights and Prospects of Potyvirus Molecular Biology," *J. Gen. Virol.* 73:1-16 (1992); and Urcuqui-Inchima et al., "Potyvirus Proteins: A Wealth of Functions," *Virus Res.* 74:157-175 (2001)), very little is understood about the identity and functions of host factors that are required for potyviral infection. Towards this end, the identification of naturally occurring host mutations that result in viral resistance and display monogenic recessive inheritance should define an important resource. The "negative model" of plant virus resistance predicts that a recessive resistance gene may represent a deleted or defective host protein that is essential for viral infection but is dispensable for the host (Fraser, "The Genetics of Plant-Virus Interactions: Implications for Plant Breeding," *Euphytica* 63:175-185 (1992)). Recessive resistance is especially prevalent for potyviruses, comprising approximately 40% of all known resistance genes (Provvidenti et al., "Sources of Resistance to Viruses in the Potyviridae," *Arch. Virol.* 5:189-211 (1992)). Many of these genes, including the *Capsicum* resistance gene *pvr1*, have been used successfully for decades in crop breeding programs as effective and stable sources of resistance (Greenleaf, "Pepper Breeding," In Basset, ed., *Breeding Vegetable Crops*, Westport, CT:AVI Pub., pp. 67-134 (1986)).

[0009]   The potyviral NIa protein, also known as VPg-Pro, is comprised of an N-terminal VPg and C-terminal protease and participates in several replicative and proteolytic functions during potyvirus infection (Revers et al., "New Advances in Understanding the Molecular Biology of Plant/Potyvirus Interactions," *Mol. Plant Microbe Interact.* 12:367-376 (1999)). The central region of VPg has been shown to be crucial in race-specific replication, cell-to-cell and long-distance movement functions in relation to recessive potyvirus resistance genes (Keller et al., "Potyvirus Genome-Linked Protein (VPg)

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Determines Pea Seed-Borne Mosaic Virus Pathotype-Specific Virulence in *Pisum sativum*," *Mol. Plant Microbe Interact.* 11:124-130 (1998); Masuta et al., "A Single Amino Acid Change in Viral Genome-Associated Protein of Potato Virus Y Correlates with Resistance Breaking in 'Virgin A Mutant' Tobacco,"

5   *Phytopathology* 89:118-123 (1999); Nicolas et al., "Variations in the VPg Protein Allow a Potyvirus to Overcome va Gene Resistance in Tobacco," *Virology* 237:452-459 (1997); Rajamaki et al., "Viral Genome-Linked Protein (VPg) Controls Accumulation and Phloem-Loading of a Potyvirus in Inoculated Potato Leaves," *Mol. Plant Microbe Interact.* 15:138-149 (2002); and Schaad et al.,

10 "VPg of Tobacco Etch Potyvirus is a Host Genotype-Specific Determinant for Long-Distance Movement," *J. Virol.* 71:8624-8631 (1997)). The importance of NIa in potyvirus replication and movement has also prompted studies to identify host factors that interact with this protein using *in vitro* interaction assays. One study showed strong interaction in yeast two-hybrid assays between *Tobacco etch*

15 *virus* (TEV) NIa and translation initiation factor eIF4E isolated from tomato and tobacco (Schaad et al., "Strain-Specific Interaction of the Tobacco Etch Virus NIa Protein with the Translation Initiation Factor eIF4E in the Yeast Two-Hybrid System," *Virology* 273:300-306 (2000)). Strong interactions have also been observed between *Arabidopsis thaliana* eIF4E or eIF(iso)4E and *Turnip mosaic*

20 *virus* (TuMV) VPg-Pro both in yeast two-hybrid and ELISA-based *in vitro* binding assays (Wittmann et al., "Interaction of the Viral Protein Genome Linked of Turnip Mosaic Potyvirus with the Translational Eukaryotic Initiation Factor (iso) 4E of *Arabidopsis thaliana* Using the Yeast Two-Hybrid System," *Virology* 234:84-92 (1997)). Furthermore, the interaction of *Arabidopsis* eIF(iso)4E and

25 TuMV VPg-Pro correlated with viral infectivity (Leonard et al., "Complex Formation Between Potyvirus VPg and Translation Eukaryotic Initiation Factor 4E Correlates with Virus Infectivity," *J. Virol.* 74:7730-7737 (2000)).

[0010]   The present invention is directed to overcoming these deficiencies in the art.

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SUMMARY OF THE INVENTION

[0011]   The present invention relates to a method of imparting virus resistance to plants. This method involves silencing a gene encoding a translation initiation factor eIF4E in the plant under conditions effective to impart virus resistance to the plant.

[0012]   The present invention also relates to a genetic construct containing a nucleic acid molecule which silences a gene encoding a translation initiation factor eIF4E in a plant. The genetic construct further contains a plant promoter heterologous to the nucleic acid molecule. The geneic construct also contains a terminator. The plant promoter and the terminator are operatively coupled to the nucleic acid molecule. The present invention further relates to an expression system containing the genetic construct and a host cell transformed with the genetic construct.

[0013]   The present invention also relates to a plant transformed with a genetic construct that contains: (i) a nucleic acid molecule which silences a gene encoding a translation initiation factor eIF4E in a plant; (ii) a plant promoter; and (iii) a terminator. The plant promoter and the terminator are operatively coupled to the nucleic acid molecule. Further, either the nucleic acid molecule, the plant promoter, or the terminator are heterologous to the transformed plant. The present invention further relates to a component part of, a fruit of, and a plant seed produced from the transformed plant.

[0014]   The present invention also relates to a plant seed transformed with a genetic construct containing: (i) a nucleic acid molecule which silences a gene encoding a translation initiation factor eIF4E in a plant; (ii) a plant promoter; and (iii) a terminator. The plant promoter and the terminator are operatively coupled to the nucleic acid molecule. Further, either the nucleic acid molecule, the plant promoter, or the terminator are heterologous to the transformed plant seed.

[0015]   The present invention relates to an isolated nucleic acid molecule encoding a mutant translation initiation factor eIF4E that is effective in imparting virus resistance in plants. The nucleic acid molecule encodes a mutant translation initiation factor eIF4E having an amino acid sequence of SEQ ID NO:4 and variants thereof that are at least 95 percent similar to SEQ ID NO:4.

Alternatively, the nucleic acid molecule encodes a mutant translation initiation
factor eIF4E having an amino acid sequence of SEQ ID NO:6 and variants thereof
that are at least 95 percent similar to SEQ ID NO:6. Alternatively, the nucleic
acid molecule encodes a mutant translation initiation factor eIF4E having an

5   amino acid sequence of SEQ ID NO:8 and variants thereof that are at least 95
percent similar to SEQ ID NO:8. Alternatively, the nucleic acid molecule encodes
a mutant translation initiation factor eIF4E having an amino acid sequence that is
at least 85 percent similar to a non-mutant translation initiation factor eIF4E of
SEQ ID NO:2 and containing at least one substitution of at least one amino acid

10   residue of SEQ ID NO:2 selected from the group consisting of T51A, P66T,
V67E, K71R, L79R, G107P, and D109R. The present invention also relates to an
isolated mutant translation initiation factor eIF4E encoded by the isolated nucleic
acid molecule. The present invention further relates to a recombinant expression
system and a host cell containing the isolated nucleic acid molecule, as well as a

15   transgenic plant and a transgenic plant seed transformed with the isolated nucleic
acid molecule.

[0016]   The present invention also relates to a method of making a mutant
translation initiation factor eIF4E. This method involves growing a host cell
containing an isolated nucleic acid molecule encoding a mutant translation

20   initiation factor eIF4E of the present invention under conditions whereby the host
cell expresses the mutant translation initiation factor eIF4E. The mutant
translation initiation factor eIF4E is then isolated.

[0017]   The present invention also relates to a method of making a
transgenic plant having enhanced virus resistance compared to that of a non-

25   transgenic plant. This method involves transforming a non-transgenic plant cell
with a nucleic acid molecule encoding a mutant translation initiation factor eIF4E
of the present invention under conditions effective to yield a transgenic plant cell
having enhanced virus resistance compared to that of a non-transgenic plant. A
transgenic plant is regenerated from the transformed plant cell. The present

30   invention further relates to a transgenic plant and plant seed produced by this
method.

[0018]   The present invention also relates to a method of imparting virus
resistance to plants by providing a nucleic acid molecule encoding a heterologous

translation initiation factor eIF4E. A plant is transformed with the nucleic acid molecule under conditions effective to yield a transgenic plant that overexpresses the heterologous translation initiation factor eIF4E. This method is effective in imparting virus resistance to the transgenic plant. The present invention also

5   relates to transgenic plants produced by this method, as well as component parts, seeds, and fruits of the transgenic plant.

BRIEF DESCRIPTION OF THE DRAWINGS

10   [0019]   Figures 1A-1C demonstrate that eIF4E-687 colocalizes with *pvr1*. Figure 1A: RFLP detected with eIF4E-687 ORF probe cosegregated with susceptible (S) or resistant (R) phenotypes in an interspecific F_2 population of 75 individuals screened with PepMoV. Figure 1B: The *SstI* CAPS marker P56 co-segregated with response to TEV-HAT and PepMoV-FL in 114 *C. chinense* F_3

15   families scored resistant (R), segregating (H), or uniformly susceptible (S) to both viruses. Figure 1C: *pvr1* and *pvr2²* fail to genetically complement. Black bars: screened F_1 populations from inter-crossing *C. annuum* '5502' or '3618' (*pvr1/pvr1*) with 'Florida VR2' (VR2) or 'Dempsey' (DEMP) (*pvr2²/pvr2²*) were resistant to TEV-HAT; susceptible check, *C. annuum* 'Jupiter'. Hatched bars:

20   5502 × DEMP F_2 population was resistant to TEV-HAT; susceptible check, *C. annuum* 'Early Cal Wonder' (ECW).

[0020]   Figures 2A-2B show amino acid alignments and crystal structure comparisions of mouse eIF4E and eIF4E-687. Figure 2A: Amino acid alignment of mouse eIF4E with eIF4E-687 sequences from *Capsicum*. *Capsicum* sequences

25   were aligned with the reference *pvr1⁺* sequence *C. annuum* 'NuMex RNaky' (RN) using the Clustal algorithm of DNASTAR. Other *pvr1⁺* genotypes: *C. annuum* 'Early Cal Wonder' (ECW) and *C. chinense* 'Habanero' (HAB); *pvr1/pvr1* genotypes: *C. chinense* PI 152225 (225), PI 159236 (236), PI 159234 (234) and *C. annuum* '3618', '4074', and '5502'; *pvr1²/pvr1²* (also referred to as *pvr2²/pvr2²*)

30   genotypes: *C. annuum* 'Dempsey' (DEMP), 'DelRay Bell' (DRB) and 'Florida VR2' (VR2); *pvr1¹/pvr1¹* (also referred to as *pvr2¹/pvr2¹*) genotype: *C. annuum* 'Yolo Y' (YY). RN amino acids identical to mouse are denoted by vertical bars;

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substitutions resulting in a similar amino acid are indicated by colons. Amino acids widely conserved across kingdoms (Marcotrigiano et al., "Cocrystal Structure of the Messenger RNA 5' Cap-Binding Protein (eIF4E) Bound to 7-Methyl-GDP," *Cell* 89:951-961 (1997), which is hereby incorporated by reference

5   in its entirety) are underlined. Highly conserved cap-binding residues are colored red. Amino acid substitutions specific to *pvr1* are colored green. Amino acid substitutions shared by *pvr1*[1] and *pvr1*[2] are in yellow. The substitution specific to *pvr1*[2] is colored blue. Upper and lower rulers denote mouse and pepper amino acid position, respectively. Functional classifications are: s, stacking tryptophan;

10 d, conserved dorsal residue; g, hydrogen bonding to guanine; r, stabilizing Arg-157; p, interaction with phosphate groups of m^7GDP; m, van der Waals interaction with m^7GDP; *, site of phosphorylation. *Capsicum* amino acids identical to RN are denoted by a period; substituted residues are listed by symbol. Figure 2B: Comparison of eIF4E-687 alleles from potyvirus-susceptible and resistant

15 *Capsicum* genotypes using models derived from the crystal structure of mouse eIF4E. Space-filling (upper) and chain (lower) models depict backbones of the proteins in gray with side-chains shown only for colored residues. Highly conserved cap-binding residues are shown in red labeled with their position in the pepper protein. Left panel: homology model of eIF4E-687 from *pvr1*[+] genotype

20 *C. annuum* 'NuMex RNaky' (RN); center panel: the homology model for eIF4E-687 from *pvr1* genotypes with amino acid substitutions (green) relative to RN labeled by position; right panel, a composite homology model for eIF4E-687 showing substitutions shared by *pvr1*[1] and *pvr1*[2] (yellow) and the unique mutation in *pvr1*[2] (blue).

25 **[0021]**   Figure 3 shows transient expression of eIF4E-687-RN functionally complemented *pvr1*. *Agrobacterium* strain C58C1 cultures with and without pCAMBIA35S-eIF4E-687-RN or containing pCAMBIA-GFP grown under *vir* gene-inducing conditions and infiltrated into the intracellular spaces of the TEV-resistant pepper leaves at the 2-4 true leaf stage. Three days after infiltration,

30 crude extract from TEV-HAT infected tobacco KY14 ground in 0.05 M potassium phosphate was rub-inoculated on the same leaves. Total RNA was isolated from the inoculated leaves 3 and 8 days after inoculation and subjected to RNA blot

analysis probed with TEV VPg to detect viral genome amplification and eIF4E-687 overexpression.

[0022]   Figures 4A-4C show that the eIF4E-687-234 allele failed to interact with TEV-HAT-VPg and mRNA cap analogs *in vitro*. Figure 4A: β-galactosidase assay of yeast two-hybrid interaction between TEV-HAT VPg and alleles isolated from RN (S) and 234 (R). Bait plasmid pEG202 was used to express the fusion protein TEV-HAT VPg. The prey plasmid pJG4-5 was used to express pepper eIF4E. pEG202: Pto and PJG4-5:AvrPto were served as a positive control. Figure 4B: Cap-binding assay of pepper eIF4E-687 sequences expressed in *E. coli* from susceptible RN and resistant 234. Upper panel: Coomassie blue-stained gel of the eIF4E-GST fusion proteins. Lane 1 and 3: eIF4E fusion proteins purified by GST- Sepharose column; lane 2 and 4: eIF4e fusion proteins purified by m^7-GTP-Sepharose column; lane M: pre-stained size marker. Lower panel: corresponding immunoblot probed with human eIF4E antibody. Arrows indicate eIF4E fusion protein bands. Figure 4C: A model depicting proposed roles of eIF4E-687-VPg interaction in the cellular resistance mechanism of *pvr1* and *pvr2²*. An ER-derived membrane is shown in the upper left corner. Proteins are depicted as follows: 6K (dark gray), VPg (blue), NIa-Pro (green), NIb (brown), poly-A binding protein (PABP) (yellow), eIF4E-687 (red), and eIF4G (light gray). A positive stranded template for negative strand RNA synthesis (approximately 10kb) is depicted in black with 5' VPg attached (blue). The picornavirus *cis*-replicating element (cre) motif AAACA and poly-A tail are indicated. The viral 6K protein, prior to cleavage from VPg and NIa-Pro domains of NIa, anchors the replication complex to the ER membrane. NIa-Pro recruits NIb to prime VPg via uridylylation of a tyrosine (Murphy et al., "A Tyrosine Residue in the Small Nuclear Inclusion Protein of Tobacco Vein Mottling Virus Links the VPg to the Viral RNA," *J. Virol.* 65:511-3 (1991), which is hereby incorporated by reference in its entirety) for replication (Schaad et al., "Formation of Plant RNA Virus Replication Complexes on Membranes: Role of an Endoplasmic Reticulum-Targeted Viral Protein.," *Embo J.* 16:4049-59 (1997), which is hereby incorporated by reference in its entirety). In picornaviruses, the VPg-priming template is a *cis*-acting replicative element (cre), a stem-loop structure containing the conserved motif AAACA (Paul et al., "Identification of an RNA Hairpin in

Poliovirus RNA that Serves as the Primary Template in the *In vitro* Uridylylation of VPg," *J. Virol.* 74:10359-70 (2000), which is hereby incorporated by reference in its entirety). TEV also has 3'*cis*-required elements for replication including a prominent stem-loop structure containing AAACA (Haldeman-Cahill et al.,

5   "Secondary Structures in the Capsid Protein Coding Sequence and 3' Nontranslated Region Involved in Amplification of the Tobacco Etch Virus Genome," *J. Virol.* 72:4072-9 (1998), which is hereby incorporated by reference in its entirety). This same region in TEV contains a conserved sequence motif from a luteovirus 3' translational enhancer that has been shown to bind eIF4E and

10   eIF(iso)4E (Wang et al., "A Viral Sequence in the 3'-Untranslated Region Mimics a 5' Cap in Facilitating Translation of Uncapped mRNA," *Embo J.* 16:4107-16 (1997); and Miller et al., "The RNA World in Plants: Post-Transcriptional Control III," *Plant Cell* 13:1710-7 (2001), which are hereby incorporated by reference in their entirety). It is proposed that in potyvirus infection, eIF4E binds directly to

15   the cre site to recruit VPg to the uridylylation template. NIa-Pro then binds and stabilizes the RNA while recruiting the NIb polymerase to catalyze the uridylylation reaction similar to polio 3CDpro and 3Dpol (Paul et al., "Identification of an RNA Hairpin in Poliovirus RNA that Serves as the Primary Template in the *In vitro* Uridylylation of VPg," *J. Virol.* 74:10359-70 (2000),

20   which is hereby incorporated by reference in its entirety). A host uridylyl transferase-like protein identified in tomato as an interacting protein with TEV VPg may represent an alternate catalyst to NIb (Schaad et al., "Strain-Specific Interaction of the Tobacco Etch Virus NIa Protein with the Translation Initiation Factor eIF4E in the Yeast Two-Hybrid System," *Virology* 273:300-6 (2000),

25   which is hereby incorporated by reference in its entirety). The interaction of NIb with host PABP (Wang et al., "Interaction Between Zucchini Yellow Mosaic Potyvirus RNA-Dependent RNA Polymerase and Host Poly-(A) Binding Protein," *Virology* 275:433-43 (2000), which is hereby incorporated by reference in its entirety) brings the poly-A tail to the VPg priming site so RNA replication could

30   proceed efficiently. It is possible that eIF4E-687 and PABP may enter the replication complex bound to eIF4G. Mutant eIF4E that binds the cre site but fails to interact with VPg, or bind VPg and fails to bind the cre site, would block the replication process.

[0023]   Figures 5A-5C show a Northern blot analysis of eIF4E gene expression of transgenic lines (T0) using pBI:eIF4E RN-AS (Fig. 5A), pBI:eIF4E 234-AS (Fig. 5B), and pBI:eIF4E TAB-AS (Fig. 5C). Key: *: untransformed control. ♥: selected transgenic lines.

5   [0024]   Figure 6 shows results from an NPTII PCR reaction for testing putative transformants. Amplification confirmed putative transformants.

[0025]   Figure 7 is a graph showing an example of ELISA data from a T1 progeny silenced for eIF4E. The first two bars represent the positive (infected) and negative (uninfected) controls, respectively. The rest of the bars represent

10 individual TEV-inoculated asymptomatic transgenic segregants silenced for eIF4E. The amount of detectable viral antigen in inoculated transgenic plants is comparable to negative control treatments indicating that silencing eIF4E results in resistance to TEV.

[0026]   Figures 8A-8B demonstrate that eIF4E-687 cosegregates with *pvr1*.

15 Figure 8A: Polymorphism detected with eIF4E-687 ORF probe cosegregated with susceptible (S) or resistant (R) phenotypes in an interspecific F_2 population of 75 individuals screened with PepMoV. Susceptible parent *C. annuum* 'NuMex RNaky' (RN) and resistant parent *C. chinense* PI 159234 (234) are indicated. Figure 8B: The *Ssp*I CAPS marker P56 co-segregated with response to TEV-

20 HAT and PepMoV in 114 *C. chinense* F_3 families scored resistant (R), segregating (H), or uniformly susceptible (S) to virus infection in two independent screens. Susceptible parent 'Habanero' (HAB) and resistant parent PI 159234 (234) are indicated. M=marker lane.

[0027]   Figures 9A-9B show the results of a complementation analysis of

25 *pvr1* and *pvr2²*. Figure 9A: *pvr1* and *pvr2²* fail to genetically complement for resistance to TEV-HAT. Black bars: Screened F_1 populations from inter-crossing *C. annuum* '5502' or '3618' (both *pvr1/pvr1*) with 'Dempsey' (DEMP) or 'Florida VR2' (VR2) (both *pvr2²/pvr2²*) were resistant to TEV-HAT based on indirect ELISA absorbance readings (21 dpi). The susceptible check was *C.*

30 *annuum* 'Jupiter'. Hatched bars: The (5502 x DEMP) F_2 population was also resistant to TEV-HAT; susceptible check, *C. annuum* 'Early Cal Wonder' (ECW). Figure 9B: Functional complementation evident as TEV accumulation in *pvr1* plants by transient expression of eIF4E-RN. *Agrobacterium* LBA4404 harboring

eIF4E-234 and eIF4E-RN alleles were infiltrated into leaves of 234 plants and 3 days after agro-infiltration, TEV-HAT virus was inoculated on the leaves. Five days after TEV inoculation, inoculated leaves were harvested and subjected to immunoblot analysis with anti-eIF4E and anti-TEV antisera.

5   [0028]   Figure 10 is a two-dimensional drawing showing the structure of the eIF4E-687 locus. White boxes represent coding sequences. Black boxes represent the 28 bp 5' and 156 bp 3' untranslated regions (Ruffel et al., "A Natural Recessive Resistance Gene Against Potato Virus Y in Pepper Corresponds to the Eukaryotic Initiation Factor 4E (eIF4E)," *Plant J.* 32:1067-1075 (2002), which is

10   hereby incorporated by reference in its entirety). Translational start (ATG) and stop (TAA) sites are indicated. Locations of sequence-based *Ssp*I CAPS marker P56 (FIG. 9B) and *Mse*I CAPS marker E23 used to confirm cosegregation of eIF4E-687 with the resistance phenotype are depicted. The gray box on the left represents a 66 bp upstream sequence with 92% identity with bases 80-146 of the

15   eIF4E-687 open reading frame.

       [0029]   Figures 11A-11B are graphs showing disrupted interaction between eIF4E-687 and TEV NIa does not correlate with resistance phenotype. Figure 11A: β-galactosidase assay of yeast two-hybrid interaction between TEV NIa proteins and eIF4E-687 from RN (*pvr1*+), HAB (*pvr1*+), 234 (*pvr1*), DEMP

20   (*pvr1*²) and YY (*pvr1*¹). Bait plasmid pEG202 was used to express the fusion protein TEV-HAT NIa (upper panel), and TEV-NW NIa (lower panel), while the prey plasmid pJG4-5 was used to express pepper eIF4E-687. The empty vector pJG4-5 was used as a negative interaction control. Yeast containing known interactors pEG202: TEV-HAT NIa and PJG4-5: TEV-HAT NIb served as a

25   positive interaction control. Figure 11B: Representative response of *pvr* genotypes to TEV-HAT (upper panel) or TEV-NW (lower panel) inoculation. Indirect ELISA absorbance values (21 dpi) are indicated for the varieties ECW (*pvr1*+), RN (*pvr1*+), HAB (*pvr1*+), 234 (*pvr1*), DEMP (*pvr1*²) and YY (*pvr1*¹). ECW- represents the mock-inoculated negative control.

30   [0030]   Figure 12 shows that only the allele eIF4E-RN binds VPg-HAT and VPg-NW. Pull-down assays were conducted with GST, GST-VPgHAT, and GST-VPgNW immobilized on glutathione-Sepharose 4B beads and ³⁵S-labeled *in vitro*-translated eIF4E alleles, 234 (*pvr1*), DEMP (*pvr1*²), RN (*pvr1*+), and YY

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(*pvr1¹*). The input protein panel represents equivalent amounts of GST, GST-VPgHAT, and GST-VPgNW used in the pull-downs assay and 20% of *in vitro*-translated eIF4Es. An arrow indicates GST fusion proteins. Pull-down eluates were separated by 12% SDS-PAGE and exposed to a phosphorimager screen.

5   [0031]   Figures 13A-13B show that Cap-binding ability does not correlate with resistance among the mutant eIF4E-687 alleles. Binding assay of recombinant eIF4E proteins to m⁷-GTP-sepharose. Figure 13A: Expression of eIF4E-687 proteins in *E. coli* (DE3) and immunoblot analysis. Upper panel: Coumassie blue-stained 12% SDS-polyacrylamide gel of whole cell lysates of

10   *E.coli* before (U) and after (I) induction by the addition of 100μM IPTG. Arrows indicate the position of recombinant protein. Lower panel: Immunoblot analysis of recombinant eIF4E-687. The same amount of protein as in the upper panel was resolved by SDS-PAGE, and the expression of the proteins was monitored by immunoblot analysis using rabbit antiserum specific for human eIF4E.

15   M=molecular size marker, U=uninduced I=induced. Figure 13B: SDS-PAGE analysis of recombinant eIF4E-687 proteins after m⁷-GTP-Sepharose affinity chromatography. The soluble fraction of bacterial cell lysates was applied to the m⁷-GTP-Sepharose column and eluted with extraction buffer containing 100μM m⁷-GTP. M= molecular size marker; E1-E4: Eluted fractions using m⁷-GTP.

20   [0032]   Figure 14 shows the results of analyses of virus resistance in transgenic tomato plants containing sense eIF4E genes. Plants were inoculated and analyzed as described in Examples 34-37, *infra*. The upper panel shows dot blot analyses and the lower panel shows pictures of TEV inoculated transgenic plants. Note that progenies of eIF4E overexpressing transgenic plant, pBI::234-

25   S9, show no symptoms of TEV infection and no accumulation of TEV coat protein.

DETAILED DESCRIPTION OF THE INVENTION

[0033]   The present invention relates to a method of imparting virus
resistance to plants. This method involves silencing a gene encoding a eukaryotic
translation initiation factor 4E ("eIF4E") in the plant under conditions effective to
5   impart virus resistance to the plant.

[0034]   The gene product of the eIF4E gene is part of the eIF4F complex
(Ruffel et al., "A Natural Recessive Resistance Gene Against Potato Virus Y in
Pepper Corresponds to the Eukaryotic Initiation Factor 4E (eIF4E)," *Plant J.*
32:1067-1075 (2002), which is hereby incorporated by reference in its entirety).
10   In this complex, eIF4E provides a 5' cap-binding function during formation of
translation initiation complexes in mRNAs of eukaryotes. Silencing a virus
disease susceptible plant host's eIF4E gene can be effective in imparting virus
resistance to plants. In particular, this can be done, for example, by using
recombinant technology.

15   [0035]   A suitable gene encoding the translation initiation factor eIF4E
("eIF4E gene") that can be targeted for silencing can include, for example, any
plant eIF4E gene naturally occurring in a virus-susceptible plant. In one
embodiment, a suitable eIF4E gene can have a nucleotide sequence that is at least
70 percent similar to SEQ ID NO:1 (described, *infra*). In another embodiment, a
20   suitable eIF4E gene can have a nucleotide sequence that is at least 90 percent
similar to SEQ ID NO:1. In another embodiment, a suitable eIF4E gene can have
a nucleotide sequence that is at least 95 percent similar to SEQ ID NO:1. In yet
another embodiment, the eIF4E gene can have a nucleotide sequence that
hybridizes to SEQ ID NO:1 under stringent hybridization conditions involving,
25   for example, hybridization in a hybridization buffer containing, for example, 20
percent formamide in 0.9M saline/0.09M SSC buffer, at a temperature of about
42°C. Other suitable stringent conditions are described below.

[0036]   For the purposes of defining the level of stringency, reference can
conveniently be made to Sambrook et al., *Molecular Cloning: A Laboratory*
30   *Manual*, 2nd Edition, Cold Spring Harbor, NY, Cold Spring Harbor Laboratory
Press, at 11.45 (1989) which is hereby incorporated by reference in its entirety.
An example of low stringency conditions is 4-6X SSC/0.1-0.5% w/v SDS at 37°-

45° C for 2-3 hours. Depending on the source and concentration of the nucleic acid involved in the hybridization, alternative conditions of stringency may be employed such as medium stringent conditions. Examples of medium stringent conditions include 1-4X SSC/0.25% w/v SDS at > 45° C for 2-3 hours. An

5   example of high stringency conditions includes 0.1-1X SSC/0.1% w/v SDS at 60°C for 1-3 hours. The skilled artisan is aware of various parameters which may be altered during hybridization and washing and which will either maintain or change the stringency conditions. For example, another stringent hybridization condition is hybridization at 4X SSC at 65° C, followed by a washing in 0.1X SSC

10   at 65° C for about one hour. Alternatively, an exemplary stringent hybridization condition is in 50% formamide, 4X SSC, at 42° C. Still another example of stringent conditions include hybridization at 62° C in 6X SSC, .05X BLOTTO, and washing at 2X SSC, 0.1% SDS at 62° C.

[0037]   In one embodiment of the present invention's method of imparting

15   virus disease resistance to plants, "silencing" involves providing a transgenic plant or plant seed transformed with a "nucleic acid molecule which silences a gene encoding a translation initiation factor eIF4E." The transgenic plant, or a transgenic plant grown from the transgenic plant seed, is grown under conditions effective to impart virus resistance to the transgenic plant.

20   [0038]   As used herein, the term "a heterologous nucleic acid molecule which silences a gene encoding a translation initiation factor eIF4E" refers to a nucleic acid molecule that is not contained in the transgenic plant or plant seed prior to transformation. Fragments of such nucleic acid molecules that retain their functional ability to impart virus resistance to plants can also be used in

25   accordance with the present invention.

[0039]   A suitable nucleic acid molecule for use in the methods of the present invention can be one that encodes an amino acid sequence of SEQ ID NO:4 and/or variants thereof that are at least 95 percent similar to SEQ ID NO:4. As used herein, SEQ ID NO:4 corresponds to the amino acid sequence encoded by

30   the eIF4E-234 gene (described, *infra*).

[0040]   Another suitable nucleic acid molecule for use in the methods of the present invention can be one that encodes an amino acid sequence of SEQ ID NO:6 and/or variants thereof that are at least 95 percent similar to SEQ ID NO:6.

As used herein, SEQ ID NO:6 corresponds to the amino acid sequence encoded by the eIF4E-YY gene (described, *infra*).

[0041]   Another suitable nucleic acid molecule for use in the methods of the present invention can be one that encodes an amino acid sequence of SEQ ID NO:8 and variants thereof that are at least 95 percent similar to SEQ ID NO:8. As used herein, SEQ ID NO:8 corresponds to the amino acid sequence encoded by the eIF4E-DEMP gene (described, *infra*).

[0042]   Another suitable nucleic acid molecule for use in the methods of the present invention can be one that encodes an amino acid sequence that is at least 85 percent similar to a non-mutant translation initiation factor eIF4E of SEQ ID NO:2 and containing at least one substitution of at least one amino acid residue of SEQ ID NO:2 suitable substitutions can included, for example, T51A, P66T, V67E, K71R, L79R, G107P, and/or D109R. As used herein, SEQ ID NO:2 corresponds to the amino acid sequence encoded by the eIF4E-687 gene (described, *infra*).

[0043]   In one embodiment of the method of imparting virus resistance to plants, a suitable nucleic acid molecule can include an antisense oligonucleotide complementary to a messenger RNA encoding the translation initiation factor eIF4E. Another suitable nucleic acid molecule can include a sense oligonucleotide homologous to a messenger RNA encoding the translation initiation factor eIF4E. Yet another suitable nucleic acid molecule can include one that mediates RNA interference ("RNAi") of the gene encoding the translation initiation factor eIF4E or that mediates RNAi of a transcript of the gene. These aspects are further described herein, *infra*.

[0044]   The present invention also relates to a method of imparting virus resistance to plants by providing a nucleic acid molecule encoding a heterologous translation initiation factor eIF4E. A plant is transformed with the nucleic acid molecule under conditions effective to yield a transgenic plant that overexpresses the heterologous translation initiation factor eIF4E. In one embodiment, the nucleic acid molecule is in sense orientation. In one aspect of this method, the overexpressed heterologous translation initiation factor eIF4E causes positive interference with a plant virus. Suitable mechanisms for such positive interference includes, without limitation, inhibiting viral VPg to bind to eIF4E in

the plant, as well as inhibiting mRNA cap-binding by eIF4E. Suitable heterologous translation initiation factor eIF4Es are as described herein. The present invention also relates to transgenic plants produced by this method, as well as component parts, seeds, and fruits of the transgenic plant.

5   [0045]   The use of antisense oligonucleotides (e.g., RNA) to down-regulate the expression of specific plant genes is well known (van der Krol et al., *Nature* 333:866-869 (1988) and Smith et al., *Nature* 334:724-726 (1988), which are hereby incorporated by reference in its entirety). Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific

10 mRNA molecule (Weintraub, "Antisense RNA and DNA," *Scientific American* 262:40 (1990), which is hereby incorporated by reference in its entirety). Antisense methodology takes advantage of the fact that nucleic acids tend to pair with "complementary" sequences. By complementary, it is meant that polynucleotides are capable of base-pairing according to the standard Watson-

15 Crick rules. For example, in a target cell, the antisense nucleic acids hybridize to a target nucleic acid and interfere with transcription, and/or RNA processing, transport, translation, and/or stability. The overall effect of such interference with the target nucleic acid function is the disruption of protein expression.

[0046]   Accordingly, both antisense and sense forms of the nucleic acids of

20 the present invention are suitable for use in the DNA constructs of the invention. A single construct may contain both sense and antisense forms of one or more of the nucleic acid molecules of the present invention.

[0047]   Alternatively, a DNA construct can be configured so that the DNA molecule encodes a mRNA which is not translatable, i.e., does not result in the

25 production of a protein or polypeptide. This is achieved, for example, by introducing into the desired nucleic acid molecule sequence of the present invention one or more premature stop codons, adding one or more bases (except multiples of 3 bases) to displace the reading frame, and removing the translation initiation codon (U.S. Patent No. 5,583,021 to Dougherty et al., which is hereby

30 incorporated by reference in its entirety). This can involve the use of a primer to which a stop codon, such as TAATGA, is inserted into the sense (or "forward") PCR-primer for amplification of the full nucleic acid, between the 5' end of that primer, which corresponds to the appropriate restriction enzyme site of the vector

into which the nucleic acid is to be inserted, and the 3' end of the primer, which corresponds to the 5' sequence of the enzyme-encoding nucleic acid.

[0048]   Genes can be effective as silencers in the non-translatable antisense forms, as well as in the non-translatable sense form (Baulcombe, D.C., "Mechanisms of Pathogen-Derived Resistance to Viruses in Transgenic Plants," *Plant Cell* 8:1833-44 (1996); Dougherty, W. G., et al., "Transgenes and Gene Suppression: Telling us Something New?," *Current Opinion in Cell Biology* 7:399-05 (1995); and Lomonossoff, G.P., "Pathogen-Derived Resistance to Plant Viruses," *Ann. Rev. Phytopathol.* 33:323-43 (1995), which are hereby incorporated by reference in their entirety). Accordingly, one aspect of the present invention involves DNA constructs which contain one or more of the nucleic acid molecules of the present invention as a DNA which encodes a non-translatable mRNA, that nucleic acid molecule being inserted into the construct in either the sense or antisense orientation.

[0049]   Numerous reports have been published on critical advances in the understanding of the biochemistry and genetics of both gene silencing and RNA-interference. Similarities between RNA-interference ("RNAi") and post-transcriptional gene silencing are astonishing, and point all to the crucial role played by sequence homology in triggering these two mechanistically related phenomena (Matzke et al., "RNA-Based Silencing Strategies in Plants," *Curr. Opin. Genet. Dev.* 11(2):221-227 (2001), which is hereby incorporated by reference in its entirety). In RNAi, the introduction of double stranded RNA ("dsRNA") into animal or plant cells leads to the destruction of the endogenous, homologous mRNA, phenocopying a null mutant for that specific gene. In both post-transcriptional gene silencing and RNAi, the dsRNA is processed to short interfering molecules of 21-, 22- or 23-nucleotide RNAs ("siRNA") by a putative RNAaseIII-like enzyme (Tuschl T., "RNA Interference and Small Interfering RNAs," *Chembiochem* 2: 239-245 (2001); and Zamore et al., "RNAi: Double Stranded RNA Directs the ATP-Dependent Cleavage of mRNA at 21 to 23 Nucleotide Intervals," *Cell* 101, 25-3, (2000), which are hereby incorporated by reference in their entirety). The endogenously generated siRNAs mediate and direct the specific degradation of the target mRNA. In the case of RNAi the cleavage site in the mRNA molecule targeted for degradation is located near the

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center of the region covered by the siRNA (Elbashir et al., "RNA Interference is Mediated by 21- and 22-Nucleotide RNAs," *Gene Dev.* 15(2):188-200 (2001), which is hereby incorporated by reference in its entirety). Thus, nucleic acid molecules of the present invention that mediate RNAi can be used in the present

5   invention's method of imparting virus resistance to plants.

[0050]   Another embodiment of the method of imparting virus resistance to plants involves providing a genetic construct containing the nucleic acid molecule of the present invention. The genetic construct is then used to transform a plant cell. In another embodiment, this method further involves propagating plants

10   from the transformed plant cell. A suitable genetic construct for use in this method can further include a plant promoter and a terminator, where the plant promoter and the terminator are operatively coupled to the nucleic acid molecule. In one embodiment, the genetic construct is in an expression vector. As described in more detail, *infra*, transformation can be carried out by, without limitation,

15   *Agrobacterium*-mediated transformation, biolistic transformation, and/or electroporation. Further, suitable techniques for the above aspects of the present invention's method of imparting virus resistance to plants is described in more detail, *infra*.

[0051]   The present invention also relates to a genetic construct containing

20   a nucleic acid molecule of the present invention. The genetic construct further contains a plant promoter heterologous to the nucleic acid molecule. The geneic construct also contains a terminator. The plant promoter and the terminator are operatively coupled to the nucleic acid molecule. The present invention further relates to an expression system containing the genetic construct and a host cell

25   transformed with the genetic construct. In one embodiment, the nucleic acid molecule is in proper sense orientation. In another particular embodiment, the host cell can be, without limitation, a plant cell or a bacterial cell. Further detail regarding the genetic construct, expression system, and host cells of the present invention are described herein, *infra*.

30   [0052]   The present invention also relates to a plant transformed with a genetic construct that contains: (i) a nucleic acid molecule which silences a gene encoding a translation initiation factor eIF4E in a plant; (ii) a plant promoter; and (iii) a terminator. The plant promoter and the terminator are operatively coupled

to the nucleic acid molecule. Further, either the nucleic acid molecule, the plant promoter, or the terminator are heterologous to the transformed plant. The present invention further relates to component parts of, fruits of, and plant seeds produced from the transformed plant.

5   [0053]   The present invention also relates to a plant seed transformed with a genetic construct containing: (i) a nucleic acid molecule which silences a gene encoding a translation initiation factor eIF4E in a plant; (ii) a plant promoter; and (iii) a terminator. The plant promoter and the terminator are operatively coupled to the nucleic acid molecule. Further, either the nucleic acid molecule, the plant

10   promoter, or the terminator are heterologous to the transformed plant seed.

      [0054]   The present invention relates to an isolated nucleic acid molecule encoding a mutant translation initiation factor eIF4E that is effective in imparting virus resistance in plants. The present invention also relates to an isolated mutant translation initiation factor eIF4E encoded by the isolated nucleic acid molecule.

15   The present invention further relates to a recombinant expression system and a host cell containing the isolated nucleic molecule, as well as a transgenic plant and a transgenic plant seed transformed with the isolated nucleic acid molecule. This isolated nucleic acid molecule can be used in the present invention's method of imparting virus resistance to plants, as previously described. In one embodiment

20   of the recombinant expression system of the present invention, the isolated nucleic acid molecule is in sense orientation relative to a promoter.

      [0055]   In one embodiment, the nucleic acid molecule of the present invention encodes a mutant translation initiation factor eIF4E having an amino acid sequence of SEQ ID NO:4 and variants thereof that are at least 95 percent

25   similar to SEQ ID NO:4. SEQ ID NO:4 is the amino acid sequence of the eIF4E-234 mutant translation initation factor eIF4E (from *Capsicum chinense* PI 159234) (NCBI Accession No. AAR23918), as follows:

30   1 mataemektt tfdeaekvkl naneaddeve egeiveetdd ttsylskeia akhplehswt

       61 fwfdntvaks rqaawgsslr nvytfstved fwgaynnihh psklvvradl hcfkhkiepk

      121 wedpvcangg twkmsfskgk sdtswlytll amighqfdhe deicgavvsv rgkgekislw

35   181 tknaanetaq vsigkqwkqf ldysdsvgfi fhddakrldr naknrytv

[0056]   SEQ ID NO:4 is encoded by the eIF4E-234 nucleotide sequence of SEQ ID NO:3 (from *Capsicum chinense* PI 159234) (NCBI Accession No. AY485129), as follows:

```
  1 atggcaacag ctgaaatgga gaaaacgacg acgtttgatg aagctgagaa ggtgaaattg
 61 aatgctaatg aggcagatga tgaagttgaa gaaggtgaaa ttgttgaaga aactgatgat
121 acgacgtcgt atttgagcaa agaaatagca gcaaagcatc cattagagca ttcatggact
181 ttctggtttg ataatacagt ggcgaaatcg agacaagctg cttggggtag ctcgcttcgc
241 aacgtctaca ctttctccac tgttgaagat ttttggggtg cttacaataa tatccaccac
301 ccaagcaagt tagttgtgag agcagactta cattgtttca agcataaaat tgagccaaag
361 tgggaagatc ctgtatgtgc caatggaggg acatggaaaa tgagtttttc aaagggtaaa
421 tctgatacca gctggctata tacgctgctt gcaatgattg gacatcaatt cgatcatgaa
481 gatgaaattt gtggagcagt agttagtgtc agaggtaagg gagaaaaaat atctttgtgg
541 accaagaatg ctgcaaatga aacggctcag gttagcattg gtaagcaatg gaagcagttt
601 ctggattaca gcgacagtgt tggcttcata tttcacgacg atgcaaagag gctcgacaga
661 aatgcaaaga atcgttacac cgtatag
```

[0057]   In another embodiment, the nucleic acid molecule of the present invention encodes a mutant translation initiation factor eIF4E having an amino acid sequence of SEQ ID NO:6 and variants thereof that are at least 95 percent similar to SEQ ID NO:6. SEQ ID NO:6 is the amino acid sequence of the eIF4E-YY mutant translation initation factor eIF4E (from *Capsicum annuum* 'Yolo Y') (NCBI Accession No. AAR23919), as follows:

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  1 mataemektt tfdeaekvkl naneaddeve egeiveetdd ttsylskeia tkhplehswt
 61 fwfdnpeaks kqaawgssrr nvytfstved fwgaynnihh psklvvgadl hcfkhkiepk
121 wedpvcangg twkmsfskgk sdtswlytll amighqfdhe deicgavvsv rgkgekislw
181 tknaanetaq vsigkqwkqf ldysdsvgfi fhddakrldr naknrytv
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[0058]   SEQ ID NO:6 is encoded by the eIF4E-YY nucleotide sequence of SEQ ID NO:5 (from *Capsicum annuum* 'Yolo Y') (NCBI Accession No. AY485130), as follows:

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  1 atggcaacag ctgaaatgga gaaaacgacg acgtttgatg aagctgagaa ggtgaaattg
 61 aatgctaatg aggcagatga tgaagttgaa gaaggtgaaa ttgttgaaga aactgatgat
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121 acgacgtcgt atttgagcaa agaaatagca acaaagcatc cattagagca ttcatggact

181 ttctggtttg ataatccaga ggcgaaatcg aaacaagctg cttggggtag ctcgcgtcgc

241 aacgtctaca ctttctccac tgttgaagat ttttggggtg cttacaataa tatccaccac

301 ccaagcaagt tagttgtggg agcagactta cattgtttca agcataaaat tgagccaaag

361 tgggaagatc ctgtatgtgc caatggaggg acatggaaaa tgagtttttc aaagggtaaa

421 tctgatacca gctggctata tacgctgctt gcaatgattg gacatcaatt cgatcatgaa

481 gatgaaattt gtggagcagt agttagtgtc agaggtaagg gagaaaaaat atctttgtgg

541 accaagaatg ctgcaaatga aacggctcag gttagcattg gtaagcaatg gaagcagttt

601 ctggattaca gcgacagtgt tggcttcata tttcacgacg atgcaaagag gctcgacaga

661 aatgcaaaga atcgttacac cgtatag
```

[0059]   In another embodiment, the nucleic acid molecule of the present invention encodes a mutant translation initiation factor eIF4E having an amino acid sequence of SEQ ID NO:8 and variants thereof that are at least 95 percent similar to SEQ ID NO:8. As referred to herein, SEQ ID NO:8 is the amino acid sequence of the eIF4E-DEMP mutant translation initation factor eIF4E (from *Capsicum annuum* 'Dempsey') (NCBI Accession No. AAR23920), as follows:

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  1 mataemektt tfdeaekvkl naneaddeve egeiveetdd ttsylskeia tkhplehswt

 61 fwfdnpeaks kqaawgssrr nvytfstved fwgaynnihh psklvvganl hcfkhkiepk

121 wedpvcangg twkmsfskgk sdtswlytll amighqfdhe deicgavvsv rgkgekislw

181 tknaanetaq vsigkqwkqf ldysdsvgfi fhddakrldr naknrytv
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[0060]   SEQ ID NO:8 is encoded by the eIF4E-DEMP nucleotide sequence of SEQ ID NO:7 (from *Capsicum annuum* 'Dempsey') (NCBI Accession No. AY485131), as follows:

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  1 atggcaacag ctgaaatgga gaaaacgacg acgtttgatg aagctgagaa ggtgaaattg

 61 aatgctaatg aggcagatga tgaagttgaa gaaggtgaaa ttgttgaaga aactgatgat

121 acgacgtcgt atttgagcaa agaaatagca acaaagcatc cattagagca ttcatggact

181 ttctggtttg ataatccaga ggcgaaatcg aaacaagctg cttggggtag ctcgcgtcgc

241 aacgtctaca ctttctccac tgttgaagat ttttggggtg cttacaataa tatccaccac

301 ccaagcaagt tagttgtggg agcaaactta cattgtttca agcataaaat tgagccaaag

361 tgggaagatc ctgtatgtgc caatggaggg acatggaaaa tgagtttttc aaagggtaaa

421 tctgatacca gctggctata tacgctgctt gcaatgattg gacatcaatt cgatcatgaa
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481 gatgaaattt gtggagcagt agttagtgtc agaggtaagg gagaaaaaat atctttgtgg
541 accaagaatg ctgcaaatga aacggctcag gttagcattg gtaagcaatg gaagcagttt
601 ctggattaca gcgacagtgt tggcttcata tttcacgacg atgcaaagag gctcgacaga
661 aatgccaaga atcgttacac cgtatag
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[0061]   In another embodiment, the nucleic acid molecule encodes a mutant translation initiation factor eIF4E having an amino acid sequence that is at least 85 percent similar to a non-mutant translation initiation factor eIF4E of SEQ ID NO:2. In this embodiment, the mutant translation initiation factor eIF4E contains at least one substitution of at least one amino acid residue of SEQ ID NO:2. Suitable substitutions can include, for example, T51A, P66T, V67E, K71R, L79R, G107P, and/or D109R. As referred to herein, SEQ ID NO:2 is the amino acid sequence of the eIF4E-687 translation initation factor eIF4E (*Capsicum annuum* 'Yolo Wonder') (NCBI Accession No. AAM82190), as follows:

```
  1 mataemektt tfdeaekvkl naneaddeve egeiveetdd ttsylskeia tkhplehswt
 61 fwfdnpvaks kqaawgsslr nvytfstved fwgaynnihh psklvvgadl hcfkhkiepk
121 wedpvcangg twkmsfskgk sdtswlytll amighqfdhe deicgavvsv rgkgekislw
181 tknaanetaq vsigkqwkqf ldysdsvgfi fhddakrldr naknrytv
```

[0062]   SEQ ID NO:2 is encoded by the eIF4E-687 nucleotide sequence of SEQ ID NO:1 (from *Capsicum annuum* 'Yolo Wonder') (NCBI Accession No. AY122052), as follows:

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  1 aaagtaaaaa aagcacacag caccaacaat ggcaacagct gaaatggaga aaacgacgac
 61 gtttgatgaa gctgagaagg tgaaattgaa tgctaatgag gcagatgatg aagttgaaga
121 aggtgaaatt gttgaagaaa ctgatgatac gacgtcgtat ttgagcaaag aaatagcaac
181 aaagcatcca ttagagcatt catggacttt ctggtttgat aatccagtgg cgaaatcgaa
241 acaagctgct tggggtagct cgcttcgcaa cgtctacact ttctccactg ttgaagattt
301 ttggggtgct tacaataata tccaccaccc aagcaagtta gttgtgggag cagacttaca
361 ttgtttcaag cataaaattg agccaaagtg gaagatcct gtatgtgcca atggagggac
421 atggaaaatg agtttttcaa agggtaaatc tgataccagc tggctatata cgctgcttgc
481 aatgattgga catcaattcg atcatgaaga tgaaatttgt ggagcagtag ttagtgtcag
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541 aggtaaggga gaaaaaatat ctttgtggac caagaatgct gcaaatgaaa cggctcaggt

601 tagcattggt aagcaatgga agcagtttct ggattacagc gacagtgttg gcttcatatt

661 tcacgacgat gcaaagaggc tcgacagaaa tgcaaagaat cgttacacag tataattctt

721 gatgcaatgt cggaatataa gaaacacaat tcgtactgaa aagttgtagg cactagttta

781 gtttctcata cgataaagct tctggtttga gtaccttgtg tattggtgtt tgcactttct

841 aatcgcgtaa aattgtccgg ttttggattc aaaaa
```

[0063]   The isolated nucleic acid molecule of the present invention can be from various plant sources, including, without limitation, from *Capsicum* spp. or *Arabidopsis* spp.

[0064]   The present invention also relates to a method of making a mutant translation initiation factor eIF4E. This method involves growing a host cell containing an isolated nucleic acid molecule encoding a mutant translation initiation factor eIF4E of the present invention under conditions whereby the host cell expresses the mutant translation initiation factor eIF4E. The mutant translation initiation factor eIF4E is then isolated. Further detail regarding this method is provided herein, *infra*.

[0065]   The present invention also relates to a method of making a transgenic plant having enhanced virus resistance compared to that of a non-transgenic plant. This method involves transforming a non-transgenic plant cell with a nucleic acid molecule encoding a mutant translation initiation factor eIF4E of the present invention under conditions effective to yield a transgenic plant cell having enhanced virus resistance compared to that of a non-transgenic plant. A transgenic plant is regenerated from the transformed plant cell. The present invention further relates to a transgenic plant and plant seed produced by this method. Further detail on this aspect of the invention is provided *infra*.

[0066]   The present invention also relates to an isolated mutant translation initiation factor eIF4E encoded by the corresponding nucleic acid molecule of the present invention (described, *supra*). The isolated mutant translation initiation factor eIF4E can be recombinant. The isolated mutant translation initiation factor eIF4E can also be in pure or non-pure form. A purified protein or polypeptide of the mutant translation initiation factor eIF4E of the present invention can be obtained by several methods. The purified protein or polypeptide of the mutant

translation initiation factor eIF4E of the present invention is preferably produced in pure form (preferably at least about 80%, more preferably 90% pure) by conventional techniques well known in the art. Typically, the purified protein or polypeptide of the mutant translation initiation factor eIF4E of the present

5   invention is secreted into the growth medium of recombinant host cells. Alternatively, the purified protein or polypeptide of the mutant translation initiation factor eIF4E of the present invention is produced but not secreted into growth medium. In such cases, to isolate the protein or polypeptide of the mutant translation initiation factor eIF4E, the host cell carrying a recombinant plasmid is

10   propagated, lysed by sonication, heat, or chemical treatment, and the homogenate is centrifuged to remove cell debris. The supernatant is then subjected to sequential ammonium sulfate precipitation. The fraction containing the protein or polypeptide of the mutant translation initiation factor eIF4E of the present invention is subjected to gel filtration in an appropriately sized dextran or

15   polyacrylamide column to separate the proteins. If necessary, the protein fraction (containing the mutant translation initiation factor eIF4E of the present invention) can be further purified by high performance liquid chromatography ("HPLC").

[0067]   As previously discussed, the present invention also relates to a recombinant expression system that contains the various nucleic acid molecules of

20   the present invention. This can involve incorporating the nucleic acid molecules of the present invention into host cells using conventional recombinant DNA technology. Generally, this involves inserting the nucleic acid molecule into an expression system to which the nucleic acid molecule is heterologous (i.e., not normally present). The heterologous nucleic acid molecule is inserted into the

25   expression system which includes the necessary elements for the transcription and translation of the inserted protein coding sequences. In one embodiment, the expression system of the present invention contains the nucleic acid molecule of the present invention in proper sense orientation relative to a promoter.

[0068]   The nucleic acid molecules of the present invention can be inserted

30   into any of the many available expression vectors and cell systems using reagents that are well known in the art. Suitable vectors include, but are not limited to, the following viral vectors such as lambda vector system gt11, gt WES.tB, Charon 4, and plasmid vectors such as pBR322, pBR325, pACYC177, pACYC1084, pUC8,

pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKC101, SV 40, pBluescript II
SK +/- or KS +/- (see "Stratagene Cloning Systems" Catalog (1993) from
Stratagene, La Jolla, CA, which is hereby incorporated by reference in its
entirety), pQE, pIH821, pGEX, pET series (see Studier et al., "Use of T7 RNA

5   Polymerase to Direct Expression of Cloned Genes," *Gene Expression Technology*
vol. 185 (1990), which is hereby incorporated by reference in its entirety), and any
derivatives thereof. Recombinant molecules can be introduced into cells via
transformation, particularly transduction, conjugation, mobilization, or
electroporation. The DNA sequences are cloned into the vector using standard

10   cloning procedures in the art, as described by Sambrook et al., *Molecular
Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor, NY, Cold
Spring Harbor Laboratory Press (1989); and Ausubel et al., *Current Protocols in
Molecular Biology*, John Wiley & Sons, New York, N.Y., (1989) which are
hereby incorporated by reference in their entirety.

15   **[0069]**   In preparing a DNA vector for expression, the various DNA
sequences can normally be inserted or substituted into a bacterial plasmid. Any
convenient plasmid can be employed, which will be characterized by having a
bacterial replication system, a marker which allows for selection in a bacterium
and generally one or more unique, conveniently located restriction sites.

20   Numerous plasmids, referred to as transformation vectors, are available for plant
transformation. The selection of a vector will depend on the preferred
transformation technique and target species for transformation. A variety of
vectors are available for stable transformation using *Agrobacterium tumefaciens*, a
soilborne bacterium that causes crown gall. Crown gall are characterized by

25   tumors or galls that develop on the lower stem and main roots of the infected
plant. These tumors are due to the transfer and incorporation of part of the
bacterium plasmid DNA into the plant chromosomal DNA. This transfer DNA
(T-DNA) is expressed along with the normal genes of the plant cell. The plasmid
DNA, pTi or Ti-DNA, for "tumor inducing plasmid," contains the vir genes

30   necessary for movement of the T-DNA into the plant. The T-DNA carries genes
that encode proteins involved in the biosynthesis of plant regulatory factors, and
bacterial nutrients (opines). The T-DNA is delimited by two 25 bp imperfect
direct repeat sequences called the "border sequences." By removing the oncogene

and opine genes, and replacing them with a gene of interest, it is possible to transfer foreign DNA into the plant without the formation of tumors or the multiplication of Agrobacterium tumefaciens (Fraley et al., "Expression of Bacterial Genes in Plant Cells," *Proc. Nat'l Acad. Sci.* 80:4803-4807 (1983),

5   which is hereby incorporated by reference in its entirety).

[0070]   Further improvement of this technique led to the development of the binary vector system (Bevan, "Binary *Agrobacterium* Vectors for Plant Transformation," *Nucleic Acids Res.* 12:8711-8721 (1984), which is hereby incorporated by reference in its entirety). In this system, all the T-DNA sequences

10   (including the borders) are removed from the pTi, and a second vector containing T-DNA is introduced into *Agrobacterium tumefaciens*. This second vector has the advantage of being replicable in *E. coli* as well as *A. tumefaciens*, and contains a multiclonal site that facilitates the cloning of a transgene. An example of a commonly used vector is pBin19 (Frisch et al., "Complete Sequence of the Binary

15   Vector Bin19," *Plant Molec. Biol.* 27:405-409 (1995), which is hereby incorporated by reference in its entirety). Any appropriate vectors now known or later described for genetic transformation are suitable for use with the present invention.

[0071]   U.S. Patent No. 4,237,224 issued to Cohen and Boyer, which is

20   hereby incorporated by reference in its entirety, describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including prokaryotic organisms and eukaryotic cells grown in tissue culture.

25   [0072]   Certain "control elements" or "regulatory sequences" are also incorporated into the vector-construct. These include non-translated regions of the vector, promoters, and 5' and 3' untranslated regions which interact with host cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and host

30   utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used.

[0073]   A constitutive promoter is a promoter that directs expression of a gene throughout the development and life of an organism. Examples of some

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constitutive promoters that are widely used for inducing expression of transgenes include the nopoline synthase ("NOS") gene promoter, from *Agrobacterium tumefaciens* (U.S. Patent No. 5,034,322 issued to Rogers et al., which is hereby incorporated by reference in its entirety), the cauliflower mosaic virus ("CaMV")

5   35S and 19S promoters (U.S. Patent No. 5,352,605 issued to Fraley et al., which is hereby incorporated by reference in its entirety), those derived from any of the several actin genes, which are known to be expressed in most cells types (U.S. Patent No. 6,002,068 issued to Privalle et al., which is hereby incorporated by reference in its entirety), and the ubiquitin promoter ("ubi"), which is the promoter

10   of a gene product known to accumulate in many cell types.

[0074]   An inducible promoter is a promoter that is capable of directly or indirectly activating transcription of one or more DNA sequences or genes in response to an inducer. In the absence of an inducer, the DNA sequences or genes will not be transcribed. The inducer can be a chemical agent, such as a

15   metabolite, growth regulator, herbicide or phenolic compound, or a physiological stress directly imposed upon the plant such as cold, heat, salt, toxins, or through the action of a pathogen or disease agent such as a virus or fungus. A plant cell containing an inducible promoter may be exposed to an inducer by externally applying the inducer to the cell or plant such as by spraying, watering, heating, or

20   by exposure to the operative pathogen. An example of an appropriate inducible promoter for use in the present invention is a glucocorticoid-inducible promoter (Schena et al., " A Steroid-Inducible Gene Expression System for Plant Cells," *Proc. Natl. Acad. Sci.* 88:10421-5 (1991), which is hereby incorporated by reference in its entirety). Expression of the transgene-encoded protein is induced

25   in the transformed plants when the transgenic plants are brought into contact with nanomolar concentrations of a glucocorticoid, or by contact with dexamethasone, a glucocorticoid analog (Schena et al., " A Steroid-Inducible Gene Expression System for Plant Cells," *Proc. Natl. Acad. Sci. USA* 88:10421-5 (1991); Aoyama et al., "A Glucocorticoid-Mediated Transcriptional Induction System in

30   Transgenic Plants," *Plant J.* 11: 605-612 (1997), and McNellis et al., "Glucocorticoid-Inducible Expression of a Bacterial Avirulence Gene in Transgenic Arabidopsis Induces Hypersensitive Cell Death, *Plant J.* 14(2):247-57 (1998), which are hereby incorporated by reference in their entirety). In addition,

inducible promoters include promoters that function in a tissue specific manner to regulate the gene of interest within selected tissues of the plant. Examples of such tissue specific promoters include seed, flower, or root specific promoters as are well known in the field (U.S. Patent No. 5,750,385 issued to Shewmaker et al.,

5 which is hereby incorporated by reference in its entirety). In one embodiment of the present invention, a heterologous promoter is linked to the nucleic acid of the construct, where "heterologous promoter" is defined as a promoter to which the nucleic acid of the construct is not linked in nature.

[0075] The nucleic acid construct also includes an operable 3' regulatory

10 region, selected from among those which are capable of providing correct transcription termination and polyadenylation of mRNA for expression in the host cell of choice, operably linked to a DNA molecule which encodes for a protein of choice. A number of 3' regulatory regions are known to be operable in plants. Exemplary 3' regulatory regions include, without limitation, the nopaline synthase

15 ("nos") 3' regulatory region (Fraley et al., "Expression of Bacterial Genes in Plant Cells," *Proc. Nat'l Acad. Sci. USA*, 80:4803-4807 (1983), which is hereby incorporated by reference in its entirety) and the cauliflower mosaic virus ("CaMV") 3' regulatory region (Odell et al., "Identification of DNA Sequences Required for Activity of the Cauliflower Mosaic Virus 35S Promoter," *Nature*

20 313(6005):810-812 (1985), which is hereby incorporated by reference in its entirety). Virtually any 3' regulatory region known to be operable in plants would suffice for proper expression of the coding sequence of the nucleic acid of the present invention.

[0076] The vector of choice, suitable promoter, and an appropriate 3'

25 regulatory region can be ligated together to produce the nucleic acid construct which contains the nucleic acid molecule of the present invention, or suitable fragments thereof, using well known molecular cloning techniques as described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press (1989); Ausubel et al.,

30 *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, N.Y. (1989), which are hereby incorporated by reference in their entirety.

[0077] Once the nucleic acid construct has been prepared, it is ready to be incorporated into a host cell. Basically, this method is carried out by transforming

a host cell with the expression system of the present invention under conditions effective to yield transcription of the nucleic acid molecule in the host cell, using standard cloning procedures known in the art, such as described by Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor,

5   NY, Cold Spring Harbor Laboratory Press, (1989), which is hereby incorporated by reference in its entirety. Suitable host cells include, but are not limited to, bacterial cells, fungal cells, viral cells, yeast cells, mammalian cells, insect cells, algal cells, plant cells, and the like. Methods of transformation may result in transient or stable expression of the DNA under control of the promoter.

10 [0078]   As described, *supra*, another embodiment relates to a method of making a transgenic plant having enhanced virus resistance compared to that of a non-transgenic plant. Generally, this method involves transforming a non-transgenic plant cell with a nucleic acid molecule according to the present invention under conditions effective to yield a transgenic plant cell having

15 enhanced virus resistance compared to that of a non-transgenic plant. The transformed plant cell is then regenerated into a transgenic plant. Thus, as mentioned, *supra*, in other embodiments, the present invention includes transgenic plants and seeds produced by transformation with the nucleic acid molecule of the present invention.

20 [0079]   Further aspects of the method of making the transgenic plant of the present invention are described below.

[0080]   In one embodiment, the isolated nucleic acid of the present invention is stably inserted into the genome of the recombinant plant cell as a result of the transformation, although transient expression can serve an important

25 purpose, particularly when the plant under investigation is slow-growing. Plant tissue suitable for transformation include leaf tissue, root tissue, meristems, zygotic and somatic embryos, callus, protoplasts, tassels, pollen, embryos, anthers, and the like.

[0081]   The means of transformation chosen is that most suited to the

30 tissue to be transformed. An appropriate method of stably introducing the nucleic acid construct into plant cells is to infect a plant cell with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* previously transformed with the nucleic acid construct. As described above, the Ti (or RI) plasmid of *Agrobacterium*

enables the highly successful transfer of a foreign DNA into plant cells. Another approach to transforming plant cells involves particle bombardment (also known as biolistic transformation) of the host cell, as disclosed in U.S. Patent Nos. 4,945,050, 5,036,006, and 5,100,792, all issued to Sanford et al., and in

5   Emerschad et al., "Somatic Embryogenesis and Plant Development from Immature Zygotic Embryos of Seedless Grapes (Vitis vinifera)," *Plant Cell Reports* 14:6-12 (1995), which are hereby incorporated by reference in their entirety. Yet another method of introduction is fusion of protoplasts with other entities, either minicells, cells, lysosomes or other fusible lipid-surfaced bodies

10   (Fraley et al., *Proc. Natl. Acad. Sci. USA* 79:1859-63 (1982), which is hereby incorporated by reference in its entirety). The DNA molecule may also be introduced into the plant cells by electroporation (Fromm et al., *Proc. Natl. Acad. Sci. USA* 82:5824 (1985), which is hereby incorporated by reference in its entirety). In this technique, plant protoplasts are electroporated in the presence of

15   plasmids containing the expression cassette. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and regenerate. The precise method of transformation is not critical to the practice of the present invention. Any method that results in efficient transformation of the

20   host cell of choice is appropriate for practicing the present invention.

[0082]   After transformation, the transformed plant cells must be regenerated. Plant regeneration from cultured protoplasts is described in Evans et al., *Handbook of Plant Cell Cultures*, Vol. 1, MacMillan Publishing Co., NY (1983); and Vasil (ed.), *Cell Culture and Somatic Cell Genetics of Plants*, Acad.

25   Press, Orlando, Vol. I, 1984, and Vol. III (1986), which are hereby incorporated by reference in their entirety.

[0083]   Means for regeneration vary from species to species of plants, but generally a suspension of transformed protoplasts or a petri plate containing explants is first provided. Callus tissue is formed and shoots may be induced from

30   callus and subsequently rooted. Alternatively, embryo formation can be induced in the callus tissue. These embryos germinate as natural embryos to form plants. The culture media will generally contain various amino acids and hormones, such as auxin and cytokinins. Efficient regeneration will depend on the medium, on the

genotype, and on the history of the culture. If these three variables are controlled, then regeneration is usually reproducible and repeatable.

[0084]   Preferably, transformed cells are first identified using a selection marker simultaneously introduced into the host cells along with the nucleic acid construct of the present invention. Suitable selection markers include, without limitation, markers encoding for antibiotic resistance, such as the nptII gene which confers kanamycin resistance (Fraley et al., "Expression of Bacterial Genes in Plant Cells," *Proc. Natl. Acad. Sci. USA* 80:4803-4807 (1983), which is hereby incorporated by reference in its entirety), and the genes which confer resistance to gentamycin, G418, hygromycin, streptomycin, spectinomycin, tetracycline, chloramphenicol, and the like. Cells or tissues are grown on a selection medium containing the appropriate antibiotic, whereby generally only those transformants expressing the antibiotic resistance marker continue to grow. Other types of markers are also suitable for inclusion in the expression cassette of the present invention. For example, a gene encoding for herbicide tolerance, such as tolerance to sulfonylurea, is useful, or the dhfr gene, which confers resistance to methotrexate (Bourouis et al., *EMBO J.* 2:1099-1104 (1983), which is hereby incorporated by reference in its entirety). Similarly, "reporter genes," which encode for enzymes providing for production of an identifiable compound, are suitable. The most widely used reporter gene for gene fusion experiments has been uidA, a gene from *Escherichia coli* that encodes the β-glucuronidase protein, also known as GUS (Jefferson et al., "GUS Fusions: β Glucuronidase as a Sensitive and Versatile Gene Fusion Marker in Higher Plants," *EMBO J.* 6:3901-3907 (1987), which is hereby incorporated by reference in its entirety). Similarly, enzymes providing for production of a compound identifiable by luminescence, such as luciferase, are useful. The selection marker employed will depend on the target species; for certain target species, different antibiotics, herbicide, or biosynthesis selection markers are preferred.

[0085]   Plant cells and tissues selected by means of an inhibitory agent or other selection marker are then tested for the acquisition of the transgene by Southern blot hybridization analysis, using a probe specific to the transgenes contained in the given cassette used for transformation (Sambrook et al., *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor, NY, Cold Spring

Harbor Laboratory Press (1989), which is hereby incorporated by reference in its entirety).

[0086]   After the expression cassette is stably incorporated in transgenic plants, it can be transferred to other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed. Once transgenic plants of this type are produced, the plants themselves can be cultivated in accordance with conventional procedure so that the nucleic acid construct is present in the resulting plants. Alternatively, transgenic seeds or propagules (e.g., cuttings) are recovered from the transgenic plants. These seeds can then be planted in the soil and cultivated using conventional procedures to produce transgenic plants.

[0087]   The methods and isolated nucleic acid molecules of the present invention can be used to impart virus resistance to plants against all types of plant viruses. In one embodiment, present invention is effective in imparting virus resistance to plants against viruses of the *Potyviridae* family. In particular, the methods and isolated nucleic acid molecules can be effective in imparting virus resistance to plants against *Potyviridae* viruses such as, without limitation, Lettuce Mosaic Virus ("LMV"), Pepper Mottle Virus ("PepMoV"), Potato Virus Y ("PVY"), Tobacco Etch Virus ("TEV"), and Turnip Mosaic Virus ("TuMV").

[0088]   Examples of suitable plants, plant seeds, and/or plant cells that can be used in making or providing the transgenic plants, transgenic plant cells, and/or transgenic plant seeds of the present invention can include, without limitation, rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean, pea, chicory, lettuce, endive, cabbage, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, sugarcane, banana, mung bean, alfalfa, rye, brussel sprout, beet, parsnip citrus, *Arabidopsis*, *Saintpaulia*, petunia, pelargonium, poinsettia, chrysanthemum, carnation, and/or zinnia.

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EXAMPLES

Example 1 – **Recombinant Technology Used in Analyzing Recessive Plant Viral Resistance Due to Mutations in Translation Initiation Factor eIF4E**

[0089]   DNA and RNA blotting techniques were done by standard procedures. Mapping was done according to (Murphy et al., "Genetic Mapping of the pvr1 Locus in *Capsicum spp.* and Evidence that Distinct Potyvirus Resistance Loci Control Responses that Differ at the Whole Plant and Cellular Levels," *Mol. Plant Microbe Interact.* 11:943-951 (1998), which is hereby incorporated by reference in its entirety).

Example 2 – **Primer Sequences and PCR Cycling Procedures Used in Analyzing Recessive Plant Viral Resistance Due to Mutations in Translation Initiation Factor eIF4E**

[0090]   eIF4E-687 full-length ORF primers based on tomato eIF4E sequence (Genbank # AF259801) were as follows:

Forward 5'-atggcaacagctgaaatgg-3' (SEQ ID NO:9)

Reverse 5'-tatacggtgtaacgattcttggca-3' (SEQ ID NO:10)

[0091]   Pepper-specific sequence at the 5' and 3' ends were confirmed by RACE and PCR-Walking (Clontech, Palo Alto, CA).

[0092]   PCR cycling conditions were as follows:

95°C 3 min

(95°C 30 sec, 55°C 30 sec, 72°C 5 mins) x 1

(95°C 30 sec, 55°C 30 sec, 72°C 90 sec) x 29

72°C 10 mins

[0093]   Primers used to amplify the internal 321bp genomic sequence spanning an intron containing the *Mse*I CAPS differential cleavage site were based on conserved sequence motifs from Genbank eIF4E accessions, as follows:

Forward 5'-aatatccatcacccaag-3' (SEQ ID NO:11)

Reverse 5'-gctccacatatttcatc-3' (SEQ ID NO:12)

[0094]   PCR cycling conditions were as follows:

95°C 3 min

(95°C 30 sec, 50°C 30 sec, 72°C 5 min) x 1

(95°C 30 sec, 50°C 30 sec, 72°C 90 sec) x 29

5   72°C 10 min

[0095]   Primers used to amplify the promoter region containing the *Sst*I
CAPS differential cleavage site were based on sequence obtained by PCR-
Walking (Clontech, Palo Alto, CA). The reverse primer lies 56 bases within the
eIF4E-687 open reading frame. The following are the primers used:

10   Forward 5'-ttacacgcgccgatacacttg-3' (SEQ ID NO:13)

Reverse 5'-catcatctgccttcattagcattcaat-3' (SEQ ID NO:14)

[0096]   PCR cycling conditions were as follows:

95°C 3 min

(95°C 30 sec, 60°C 30 sec, 72°C 5 min) x 1

15   (95°C 30 sec, 60°C 30 sec, 72°C 90 sec) x 29

72°C 10 min

[0097]   Primers used to amplify eIF(iso)4E for mapping in pepper, as
follows:

Forward 5'-aacaatggccaccgaagc-3' (SEQ ID NO:15)

20   Reverse 5'-atttcacacagtatatcggctct-3' (SEQ ID NO:16)

[0098]   PCR cycling conditions were as follows:

95°C 3 min

(95°C 30 sec, 55°C 30 sec, 72°C 5 min) x 1

(95°C 30 sec, 55°C 30 sec, 72°C 90 sec) x 29

25   72°C 10 min

Example 3 – **Inoculation of Seedlings for Use in Virus Screening Procedures Used in Analyzing Recessive Plant Viral Resistance Due to Mutations in Translation Initiation Factor eIF4E**

[0099]   The first two leaves of seedlings at the 4-6 leaf stage were rub-inoculated (using carborundum) with plant extract from TEV-HAT or PepMoV infected tobacco plants ground in ice-cold 0.05 M potassium phosphate buffer, pH 8. Systemic infection was assayed at 21 days post inoculation by visual symptoms and confirmed by indirect ELISA (Voller et al., "The Detection of Viruses by Enzyme-Linked Immunosorbent Assay (ELISA)," *Journal of General Virology* 33:165-167 (1976), which is hereby incorporated by reference in its entirety).

Example 4 – **Virus Screening Procedures Used in Analyzing Recessive Plant Viral Resistance Due to Mutations in Translation Initiation Factor eIF4E**

[0100]   *Agrobacterium*-mediated transient expression, Yeast two-hybrid analysis, and Cap-binding assays were used. *Agrobacterium*-mediated transient expression assays followed the method in (Van den Ackerveken et al., "Recognition of the Bacterial Avirulence Protein AvrBs3 Occurs Inside the Host Plant Cell," *Cell* 87:1307-16 (1996), which is hereby incorporated by reference in its entirety). Yeast two-hybrid procedures followed the method in (Golemis et al., "The Yeast Two-Hybrid System: Criteria for Detecting Physiologically Significant Protein-Protein Interactions," *Curr. Issues Mol. Biol.* 1:31-45 (1999), which is hereby incorporated by reference in its entirety). Cap-binding assays followed the procedures in (Vasilescu et al., "Mutants of Eukaryotic Initiation Factor eIF-4E with Altered mRNA Cap Binding Specificity Reprogram mRNA Selection by Ribosomes in *Saccharomyces cerevisiae*," *J. Biol. Chem.* 271:7030-7 (1996), which is hereby incorporated by reference in its entirety).

Example 5 – **Amino Acid Substitutions Relating to the Analysis of Recessive Plant Viral Resistance Due to Mutations in Translation Initiation Factor eIF4E**

[0101]   Amino acid alignments of eIF4E-687 (Fig. 2A) reveal identical sequences for three *C. chinense* accessions known to carry *pvr1* and three *pvr1/pvr1 C. annuum* bell pepper breeding lines derived from a resistant *C.*

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chinense parent. Promoter sequences for the breeding lines also carry distinct sequence identical to the *C. chinense* accessions, further indicating an introgression event. There are four mutations relative to the consensus *C. annuum* susceptible sequence of RNaky and ECW. Three of the mutations change the

5   amino acid group, while the K71R mutation is conservative (positively charged polar group) and is shared by the TEV and PepMoV susceptible *C. chinense* cultivar Habanero and may be a species-specific difference. T51R represents a shift from polar to nonpolar, P66T represents a shift from nonpolar to polar, and G107R represents a shift from nonpolar to positively charged polar amino acids.

10   The combined effects of these amino acid substitutions are a conformational shift in the string of amino residues that flank the cap-binding slot of the protein (Fig. 2B). This can clearly be seen with the position of the K71R mutation. Though none of the amino acid positions mutated in the *pvr1* mutant are strictly conserved for cap-binding, the G107R mutation could interrupt the salt bond stabilization of

15   arginine 171 by aspartic acid 109, due to the introduction of another positively charged side-chain. There could also be steric hindrance based on the conformation of the G107R mutation in reference to how the cap needs to be stabilized by arginine 171. The T51A mutation in the "tail" of the protein (Fig. 2) may be involved in cap-binding also as mutations in human in histidine residues at

20   corresponding position 48 abolished cap binding (Morino et al., "Analysis of the mRNA Cap-Binding Ability of Human Eukaryotic Initiation Factor-4E by Use of Recombinant Wild-Type and Mutant Forms," *Eur. J. Biochem.* 239:597-601 (1996), which is hereby incorporated by reference in its entirety).

       [0102]   The *pvr2*[1] and *pvr2*[2] sequences share two (V67E and L79R) out of

25   their three mutations relative *to C. annuum* susceptible cultivars. The V67E mutation represents a shift from nonpolar to negatively charged polar and the L79R represents a shift from nonpolar to positively charged polar amino acids. Though the mutated residues are not directly involved in cap-binding, once again a conformation shift is seen along the string of amino acid residues flanking the

30   cap-binding slot. Finally, the *pvr2*[2] mutant has a specific D109R mutation that directly affects a highly conserved residue required for cap-binding. The D109R mutation represents a shift from negatively charged polar to uncharged polar

residues. This would interrupt the salt bond D109 ordinarily makes with

negatively charged R171 to stabilize its position for cap-binding function.

[0103]   Looking at the overall combination of point mutations, the *pvr1*

G107R mutation and *pvr2²* D109R mutations within the cap-binding slot may

5   have similar effects on cap-binding, and result in their commonly held resistance

to TEV-HAT and PVY-1. Conformational differences resulting from the two

unique mutations in each in the region flanking the cap-binding slot allele (P66T

and K71R in *pvr1* and V67E and L79R in *pvr2²*) may have similar consequences

for resistance to PVY-0. Finally, the combined effects in *pvr1* of the T51A

10   mutation in addition to the other three mutations may serve to extend the

resistance spectrum to PepMoV and PVY-1, 2 resistance. Mechanistically, *pvr1*

and *pvr2-2* appear to share a common mechanism with regard to resistance to

TEV-HAT (replication), and it is unknown whether all three alleles also share a

common mechanism for resistance to PVY-0 (cell-to-cell movement).

15

Example 6 –   Data Demonstrating that the Silencing of eIF4E Results in
Resistance to Plant Viruses

[0104]   This experiment demonstrates that when a copy of the eukaryotic

20   translation initiation factor, eIF4E, is expressed in a susceptible plant such that the

plant's own copy of the gene is silenced, that plant is then less susceptible or not

susceptible to viral infection. This experiment shows the effect when the gene is

expressed transiently, but the same effect could be created using other stable

silencing methods such as RNAi.

25   [0105]   For transient expression assays, one homolog of the gene eIF4E

from *Capsicum annuum* RNaky (susceptible to tobacco etch virus) (Potyviridae)

was cloned into pPVX201 provided by D. Baulcombe. The 687-bp coding region

gene was amplified using the primers 5'-<u>ATCGAT</u>GGCAACAGCTGAAATGG-

3' (SEQ ID NO:17) (introduced *Cla*I site is underlined) and 5'-

30   CTATACGGTCTAACGATTCT-3' (SEQ ID NO:18). Amplified fragments were

cloned into pGEM^T (Promega, Madison, WI) and confirmed by sequencing.

Products were digested with *Cla*I and *Sal*I and subcloned into corresponding

vector sites. The resulting pPVX201 derivatives were designated

pPVX201::eIF4E-RN. A derivative of pPVX201 carrying no insert was used as a

control. To increase inoculum prior to experiments, each leaf of 4 – 6 week old *N. benthamiana* seedlings was mechanically inoculated with ≥50 ug of plasmid in a 15 % bentonite suspension in 43 mM sodium phosphate buffer (pH 7.0). Systemically infected *N. benthamiana* plants inoculated with pPVX::eIF4E-RN or

5   pPVX201 developed chlorosis and systemic mosaic 14 days post-inoculation (dpi). To prepare inoculum for *Capsicum* seedlings, non-necrotic symptomatic *N. benthamiana* leaves 10 – 14 dpi were homogenized in 50 mM phosphate buffer (pH 7.0).

[0106]   Six to eight week old seedlings of four pepper genotypes

10   previously determined to be susceptible to PVX, *C. chinense* PI159234 (resistant to TEV) and *C. annuum* ECW were mechanically inoculated with one of the two constructs above, one containing eIF4E, one not. Infection was confirmed by positive indirect enzyme-linked immunosorbent assay (ELISA) and hybridization analysis. Three weeks after PVX inoculation which introduced the gene used to

15   silence eIF4E, crude extract from the test virus, TEV-HAT from infected tobacco KY14 ground in 0.05 M potassium phosphate, was rub-inoculated on upper leaves which didn't show any symptom.

[0107]   Both genotype, PI159234 and ECW, infected with pPVX201 showed typical PVX symptoms (mottling, chlorosis, distortion and spreading

20   necrosis on inoculated leaves) that moved systemically throughout the plant. When inoculated with pPVX::eIF4E-RN, the vector carrying the silencing construct, C. chinense PI159234 plants developed HR like necrotic response 10 days after inoculation and symptoms did not spread systemically. Occasionally (1 out of 10 plants in a typical experiment) an individual plant showed systemic

25   infection but symptoms were usually milder than wild type PVX symptom. In contrast, C. annuum ECW inoculated with the same virus didn't show any necrotic response on inculated leaves and showed mottling, chlorosis, and rarely necrosis, and the symptom spread systemically but very slowly.

[0108]   It is assumed that the reduced symptoms of the plants inoculated by

30   the PVX expression eIF4E-RN were affected by the gene silencing of eIF4E. Therefore, tests were performed to determine whether the pPVXeIF4E-RN infected C. annuum ECW plants, a pepper variety known to be extremely

susceptible to the TEV, can support TEV virus replication after inoculation PVX expressing eIF4E-RN.

[0109]   To test this, the TEV-susceptible genotype previously inoculated with either pPVX201 (lacking the gene) or pPVXeIF4E-RN was challenged. Two weeks after TEV inoculation, upper leaves of the infected plants were collected and tested for virus accumulation by ELISA. ELISA analysis showed significant difference in TEV accumulation between the plants infected with pPVX201 and pPVXeIF4E-RN, 1.5+/-1.0 and 0.6+/-0.27 respectively.

[0110]   Plants inoculated with the construct lacking eIF4E were infected while plants inoculated with the silencing construct were not. These data establish that viral symptoms apparent at the whole plant level and virus replication/accumulation in plant tissue can be reduced or eliminated by expressing a construct that silences eIF4E in plants.

Example 7 –   Data Demonstrating That The Transgenic Silencing Of The Plant Gene eIF4E Results In Stable Heritable Resistance To Plant Viruses

[0111]   It was demonstrated that when a copy of the eukaryotic translation initiation factor, eIF4E, is expressed in a susceptible plant such that the plant's own copy of the gene is silenced, that plant is then less susceptible or not susceptible to viral infection. This experiment demonstrated the concept that stable silencing of a plant gene can result in resistance to a plant pathogen. This is believed to be the first demonstration of this concept. Preliminary evidence suggests that silencing eIF4E will result in resistance to at least 3 viruses and perhaps more, and also that silencing a related gene eIF(iso)4E should also result in virus resistance. Silencing other host translation factors, eIF4G, eIF4F and their isoforms will likely also have an effect.

[0112]   Constructs that express the eukaryotic translation factor, eIF4E, in an anti-sense orientation were transformed into a tomato susceptible to the plant potyvirus, tomato etch virus. Control treatments included untransformed tomatoes and tomatoes of the same genotype transformed with an empty vector. The resulting transformants, the T0 generation, were analyzed for presence of the respective transgene and degree of eIF4E silencing reflected by reduced levels of

eIF4E mRNA. T0 plants were selected that showed strong silencing of this gene and were self-pollinated to generate R1 progenies. These progenies should be segregating for the transgene and therefore for resistance to the virus, if the silencing results in plant-level virus resistance.

5   [0113]   These progenies were inoculated with the virus TEV and symptoms were allowed to develop. The titer of the virus in tomato tissue was then analyzed in each plant using the assay, enzyme-linked immunosorbent assay (ELISA), which quantifies presence of the viral coat protein, using an antibody that specifically recognizes this protein. In T1 generation progenies produced by

10   T0 plants showing the strongest silencing (see Figure 1) segregating for presence of the transgene resulted in plants showing no symptoms of the disease, in contrast to positive controls (untransformed, transformed with empty vector or irrelevant gene) and plants that did not carry the transgene (Figure 2). When these plants were analyzed quantitatively using ELISA, symptomless plants that carried the

15   transgene showed low ELISA values, equivalent to negative controls, while symptomatic plants showed high ELISA values, equivalent to positive controls (Table 1). This experiment proves that by silencing a host gene transgenically, stable heritable plant disease resistance can be engineered.

[0114]   It is believed that this is the first demonstration of this principal in

20   plants. This knowledge should allow one to engineer a broad spectrum resistant response to a wide array of pathogens. These progenies are being tested further for additional viruses and the progenies of resistant plants will also be tested to confirm resistance in the T2 generation.

25   <u>**Example 8**</u> – **Recessive Plant Viral Resistance Results from Mutations in Translation Initiation Factor eIF4E**

[0115]   Three widely used recessive *Capsicum* alleles, *pvr1*, *pvr2*[1] and *pvr2*[2] confer resistance to three *Potyvirus* species, potato virus Y (PVY), pepper mottle virus (PepMoV) and tobacco etch virus (TEV). The allele *pvr1* confers

30   resistance to PVY, PepMoV and most TEV isolates; *pvr2*[1] confers resistance to PVY-0 and is allelic for this resistance with *pvr2*[2], which also controls additional PVY and TEV isolates. Both *pvr1* and *pvr2*[2] share a common resistance mechanism (blocked replication) to at least one TEV isolate, TEV-HAT (Murphy

et al., "Genetic Mapping of the pvr1 Locus in *Capsicum spp.* and Evidence that
Distinct Potyvirus Resistance Loci Control Responses that Differ at the Whole
Plant and Cellular Levels," *Mol. Plant Microbe Interact.* 11:943-951 (1998); and
Deom et al., "Resistance to Tobacco Etch Virus in *Capsicum annuum*: Inhibition

5   of Virus RNA Accumulation," *Mol. Plant Microbe Interact.* 10:917-921 (1997),
which are hereby incorporated by reference in their entirety); *pvr2*[1] blocks cell-to-
cell movement of PVY-0 (Arroyo et al., "Impaired Cell-to-Cell Movement of
Potato Virus Y in Pepper Plants Carrying the Y-A (pr2-1) Resistance Gene," *Mol.
Plant Microbe Interact.* 9:314-318 (1996), which is hereby incorporated by

10 reference in its entirety). Although the viral avirulence determinants in this
system remain unknown, the central region of the viral VPg protein has been
identified as an avirulence determinant in nearly every recessive potyvirus
resistance system studied to date (Rajamaki et al., "Viral Genome-Linked Protein
(VPg) Controls Accumulation and Phloem-Loading of a Potyvirus in Inoculated

15 Potato Leaves," *Mol. Plant Microbe Interact.* 15:138-49 (2002), which is hereby
incorporated by reference in its entirety). The host translation factors eIF4E and
eIF(iso)4E have shown strong *in vitro* interaction with VPg that in *Arabidopsis*
have been correlated with viral infectivity (Leonard et al., "Complex Formation
Between Potyvirus VPg and Translation Eukaryotic Initiation Factor 4E

20 Correlates with Virus Infectivity," *J. Virol.* 74:7730-7 (2000); and Schaad et al.,
"Strain-Specific Interaction of the Tobacco Etch Virus NIa Protein with the
Translation Initiation Factor eIF4E in the Yeast Two-Hybrid System," *Virology*
273:300-6 (2000), which are hereby incorporated by reference in their entirety);
therefore, these genes were considered candidates for the *pvr* loci.

25 **[0116]**   A hybridization probe specific to the 687bp eIF4E sequence
(eIF4E-687) (see Examples 1-5, *supra*) identified an RFLP that mapped precisely
to *pvr1* (Murphy et al., "Genetic Mapping of the pvr1 Locus in *Capsicum spp.* and
Evidence that Distinct Potyvirus Resistance Loci Control Responses that Differ at
the Whole Plant and Cellular Levels," *Mol. Plant Microbe Interact.* 11:943-951

30 (1998), which is hereby incorporated by reference in its entirety) (Fig. 1A).
Sequence-based CAPS markers confirmed this result (Fig. 1B) (see Examples 1-5,
supra). Pepper eIF(iso)4E (see Examples 1-5, *supra*) was unlinked to *pvr1*,
localizing instead to a chromosomal interval containing the recessive resistance

gene, *pvr6*, reported to act in tandem with *pvr2²* to confer resistance to chili veinal mottle potyvirus (Caranta et al., "A Complementation of Two Genes Originating from Susceptible *Capsicum annuum* Lines Confers a New and Complete Resistance to Pepper Veinal Mottle Virus," *Phytopathology* 86:739-743 (1996),

5   which is hereby incorporated by reference in its entirety). Two other eIF4E homologs did not map near known resistance genes and lacked characteristic polymorphism between resistant and susceptible genotypes. eIF4E cap-binding motifs were highly conserved among all eIF4E isoforms and homologs, implying functional redundancy within the pepper genome.

10   [0117]   Originally described as alleles of the same locus, *pvr1* and *pvr2* have since been attributed to distinct loci without clear justification. When genetic complementation for resistance to TEV-HAT was assessed, *pvr1* and *pvr2²* failed to complement (Fig. 1C) (see Examples 1-5, *supra*), defining eIF4E-687 as a candidate for all three genes.

15   [0118]   Amino acid alignments were used to compare eIF4E-687 alleles from a wide array of germplasm representing *pvr1*, *pvr2¹* and *pvr2²* (Fig. 2A). Each allele showed characteristic amino acid substitutions consistent with known breeding pedigrees. Based on these sequences, it appears *pvr1* and *pvr2* are the result of independent mutations that originated in *C. chinense* and *C. annuum*,

20   respectively. Predicted structural models show conformational shifts that could disrupt cap-binding in all three alleles and amino acid substitutions at (*pvr2²*) or near (*pvr1*) a highly conserved stabilizing residue involved in cap-binding (Marcotrigiano et al., "Cocrystal Structure of the Messenger RNA 5' Cap-Binding Protein (eIF4E) Bound to 7-Methyl-GDP," *Cell* 89:951-61 (1997), which is

25   hereby incorporated by reference in its entirety) (Fig. 2B) (see Examples 1-5, *supra*).

[0119]   Transient expression of a susceptible eIF4E-687 allele in resistant *pvr1* plant leaves via *Agrobacterium* infiltration functionally complemented the resistant phenotype (Fig. 3) (see Examples 1-5, supra). Taken together, functional

30   complementation, co-segregation, genetic complementation analyses and sequence data confirm that *pvr1*, and by extension, *pvr2* are mutant alleles of eIF4E-687.

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[0120]   Based on the reported correlation of eIF4E/VPg interaction and viral infectivity, eIF4E-687 alleles from resistant and susceptible hosts were cloned and analyzed in the yeast two-hybrid system for interaction with TEV-HAT VPg (Fig. 4A) (see Examples 1-5, *supra*). The fusion protein from the

5   susceptible host showed strong interaction with TEV-HAT VPg, while the *pvr1* fusion protein failed to interact. This result suggests that potyvirus resistance conferred by *pvr1* may be a consequence of defect(s) that interfere with the interaction between host eIF4E and viral VPg.

[0121]   In order to determine whether the *pvr1*/eIF4E-687 allele was

10   functional in its cellular role of cap-binding, the same alleles were expressed in *E. coli* and resulting proteins were assayed for binding-activity on m^7-GTP cap-analog columns (Fig. 4B) (see Examples 1-5, *supra*). eIF4E from the susceptible host showed strong cap-binding activity, while the *pvr1* product failed to bind the column. Plants homozygous for *pvr1* are phenotypically normal in every respect,

15   therefore the cap-binding activity of eIF4E-687 appears to be non-essential for host survival. Taken together, VPg and cap-binding results indicate that a functional eIF4E-687 is essential for pathogenesis of viral genotypes controlled by these genes but dispensable for the host.

[0122]   An explanation regarding the molecular basis of resistance in this

20   system must account for different resistance mechanisms (replication vs. cell-to-cell movement) attributed to this locus, and for the observation that putative structural differences in eIF4E-687 correlate with differences in strain-specificity (see Examples 1-5, *supra*). These differences would be coupled with virus strain-specific structural differences in the VPg molecule to produce an interaction

25   phenotype that determines resistance or susceptibility. The VPg/eIF4E interaction has been proposed to confer a translational advantage to the virus (Lellis et al., "Loss-of-Susceptibility Mutants of *Arabidopsis thaliana* Reveal an Essential Role for eIF(iso)4E During Potyvirus Infection," *Curr. Biol.* 12:1046-51 (2002), which is hereby incorporated by reference in its entirety), however there is evidence that

30   both eIF4E and VPg are dispensable for cap-independent translation in both potyvirus and in polio, a member of *Picornaviridae* which share many features of genome structure and function with the *Potyviridae* (Gallie, "Cap-Independent Translation Conferred by the 5' Leader of Tobacco Etch Virus is Eukaryotic

Initiation Factor 4G Dependent," *J. Virol.* 75:12141-52 (2001); Sachs et al., "Starting at the Beginning, Middle, and End: Translation Initiation in Eukaryotes," *Cell* 89:831-8 (1997); Nomoto et al., "The 5'-Terminal Structures of Poliovirion RNA and Poliovirus mRNA Differ Only in the Genome-Linked

5   Protein VPg," *Proc. Natl. Acad. Sci. USA* 74:5345-9 (1977); Keller et al., "Potyvirus Genome-Linked Protein (VPg) Determines Pea Seed-Borne Mosaic Virus Pathotype-Specific Virulence in *Pisum sativum*," *Mol. Plant Microbe Interact.* 11:124-30 (1998); and Riechmann et al., "Highlights and Prospects of Potyvirus Molecular Biology," *J. Gen. Virol.* 73:1-16 (1992), which are hereby

10   incorporated by reference in their entirety). In many RNA virus/host systems, cellular translational machinery is recruited into the viral replicase complex, bringing 5' and 3' regulatory elements together (Lai, "Cellular Factors in the Transcription and Replication of Viral RNA Genomes: A Parallel to DNA-Dependent RNA Transcription," *Virology* 244:1-12 (1998), which is hereby

15   incorporated by reference in its entirety); in poliovirus, translation and replication are coupled processes (Novak et al., "Coupling Between Genome Translation and Replication in an RNA Virus," *Genes Dev.* 8:1726-37 (1994), which is hereby incorporated by reference in its entirety).

[0123]   Based on these findings, a model that focuses on RNA replication

20   to explain how eIF4E mutants that fail to interact with VPg result in the extreme cellular resistance observed in *pvr1* and *pvr2*[2] genotypes is favored (Fig. 4C). This model predicts that mutations that disrupt eIF4E-687 binding with either VPg or the putative cre element would block replication at a very early stage and produce a phenotype consistent with *pvr1* and *pvr2*[2]. This hypothesis is based on

25   data drawn both from polio and potyvirus systems, which show sequence and structural similarity among their replication proteins (Riechmann et al., "Highlights and Prospects of Potyvirus Molecular Biology," *J. Gen. Virol.* 73:1-16 (1992), which is hereby incorporated by reference in its entirety).

[0124]   In contrast to the extreme block described above, isolated

30   protoplasts from plants homozygous for *pvr2*[1] accumulate virus to susceptible levels, suggesting that this allele should not have the functional defect above, however, eIF4E-VPg binding properties may be altered in such a way to affect VPg availability and/or function (Arroyo et al., "Impaired Cell-to-Cell Movement

of Potato Virus Y in Pepper Plants Carrying the Y-A (pr2-1) Resistance Gene,"
Mol. Plant Microbe Interact. 9:314-318 (1996), which is hereby incorporated by
reference in its entirety). VPg and the 5' terminus of the viral genome are critical
for virion assembly (Wu et al., "Evidence that Assembly of a Potyvirus Begins

5   Near the 5' Terminus of the Viral RNA," *J. Gen. Virol.* 79:1525-9 (1998), which
is hereby incorporated by reference in its entirety) and association with the coat
protein is essential for potyvirus movement (Revers et al., "New Advances in
Understanding the Molecular Biology of Plant/Potyvirus Interactions," *Mol. Plant
Microbe Interact.* 12:367-376 (1999), which is hereby incorporated by reference

10   in its entirety).

[0125]   The recent identification of eIF(iso)4E as the gene responsible for
TEV and TuMV replication-defective mutants in *Arabidopsis* (Lellis et al., "Loss-
of-Susceptibility Mutants of *Arabidopsis thaliana* Reveal an Essential Role for
eIF(iso)4E During Potyvirus Infection," *Curr. Biol.* 12:1046-51 (2002), which is

15   hereby incorporated by reference in its entirety) is complementary evidence that
cap-binding proteins provide indispensable function for potyvirus infection.   It is
now clear that both eIF4E and eIF(iso)4E are strong candidates for a number of
naturally occurring recessive potyvirus resistance genes. Based on mapping data
in tomato and pepper, *pot-1* and *pvr5* are likely to be eIF4E-687 homologs

20   (Parella et al., "Recessive Resistance Genes Against Potyviruses are Localized in
Colinear Genomic Regions of the Tomato (Lysopersicon spp.) and Pepper
(*Capsicum spp.*) Genomes," *Theor. Appl. Genet.* 105:855-861 (2002), which is
hereby incorporated by reference in its entirety), while *pvr6* is likely to be
eIF(iso)4E.

25   [0126]   In other recessive potyvirus/host systems, candidate gene analysis
will reveal the universality of this type of mutant (Keller et al., "Potyvirus
Genome-Linked Protein (VPg) Determines Pea Seed-Borne Mosaic Virus
Pathotype-Specific Virulence in *Pisum sativum*," *Mol. Plant Microbe Interact.*
11:124-30 (1998); Nicolas et al., "Variations in the VPg Protein Allow a Potyvirus

30   to Overcome va Gene Resistance in Tobacco," *Virology* 237:452-9 (1997); and
Drijfhout et al., *Netherlands Journal of Plant Pathology* 84:13-26 (1978), which
are hereby incorporated by reference in their entirety). Another possible candidate
gene for recessive resistance is PABP (Wang et al., "Interaction Between Zucchini

Yellow Mosaic Potyvirus RNA-Dependent RNA Polymerase and Host Poly-(A) Binding Protein," *Virology* 275:433-43 (2000), which is hereby incorporated by reference in its entirety), if there is sufficient redundancy in the host genome to avoid lethality. The apparently dispensable eIF4E homolog, eIF4E-687, may then define an opportunity to engineer extreme cellular-level resistance by targeted silencing in transgenic plants (Azevedo et al., "The RAR1 Interactor SGT1, an Essential Component of R Gene-Triggered Disease Resistance," *Science* 295:2073-6 (2002), which is hereby incorporated by reference in its entirety). As more is learned that links functional shifts in resistance mechanism and specificity to defined changes in interacting host and pathogen molecules, the *pvr* system should offer a unique opportunity to extend the understanding of the role host gene products play in viral pathogenesis, and the molecular determinants of host-pathogen specificity. Further, this study establishes eIF4E and eIF(iso)4E as viable candidates for a plethora of naturally occurring virus resistance genes for which molecular markers may be very useful in agriculture and may open the prospect for superior transgenic strategies to engineer highly durable resistance to control an extremely broad set of plant viruses.

Example 9 – Plant Material

[0127]   Micro-tom (Ball Seeds) were used. T0 generation transgenic plants were used for Northern blotting. T1 generation progenies of the transgenic plants were used for further analysis.

Example 10 – Plasmid Construction

[0128]   To make antisense and sense constructs of eIF4E, full length eIF4E cDNA sequences were amplified tomato and pepper cDNA using primers eIF4E-SacI/eIF4E-SmaI or eIF4E-SmaI/eIF4E-SacI and cloned into corresponding sites of pBI121 vector. Different pepper genotypes (234, RN, TAB0 representing 3 spp. of *Capsicum* were used as a source for the eIF4E allele.

[0129]   To make a RNAi construct the tomato eIF4E cDNA sequence was PCR amplified using primers containing attB1 and attB2 sequence and the PCR

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product was cloned into pHELLSGATE2 vector by BP recombination reaction as described in manufacturer's instruction.

Example 11 – Plant Transformation

5   [0130]   *Agrobacterium tumefaciens* LBA4404 was used for plant transformation. The transformation procedure was as described previously (Micro-tom transformation procedure).

Example 12 – RNA Isolation And Northern Blot Analysis

10   [0131]   Total RNA was isolated from 6 week old pepper seedling leaves using TRIZOL (Gibco BRL Life Technologies, Rockville, MD) according to the manufacturer's instruction. About 10 μg of the total RNA was separated on a 1.2% formaldehyde denaturing gel and transferred to nylon membranes. The blots were hybridized with random-primed ^{32}P labeled DNA probes as previously

15   described (Livingstone et al., "Genome Mapping in *Capsicum* and the Evolution of Genome Structure in the Solanaceae," *Genetics* 152:1183-1202 (1999), which is hereby incorporated by reference in its entirety).

Example 13 – Genomic DNA Isolation and NPTII PCR

20   [0132]   Tomato genomic DNA was isolate accoreding to standard methods and the presence of the NPTII gene was confirmed using the polymerase chain reaction with NPTII-specific primers.

Example 14 – Virus inoculation and ELISA

25   [0133]   The first two leaves of greenhouse grown seedlings at the 4-6 leaf stage were inoculated using carborundum with plant sap from TEV-HAT-infected tobacco plants ground in ice-cold 0.05 M potassium phosphate buffer, pH 8. Systemic infection was assayed at 15 days post-inoculation (dpi) by visual symptoms and confirmed by indirect ELISA. Table 1 summarizes the

30   transformation experiments resulting in resistance.

Table 1 – Summary of transformation experiments resulting in resistance

Plasmid Constructs	Total No. of Transformants	No. ofTransformansts (T0) showing low eIF4E expression	No of lines showed TEVresistance
pBI121: eIF4E-AS234	37	9	2 (234-AS27, 32)
pBI121: eIF4E-ASRN	38	3	1 (RN-AS20)
pBI121: eIF4E-ASTAB	31	6	2 (TAB-AS5, 24)
pBI121: eIF4E-234(S)	13	3	N/A
pBI121: eIF4E-RN(S)	14	3	1 (RN-S13)
pBI121: eIF4E-TAB(S)	8	1	N/A
pHELLSGATE2:TAB	20	N/A	N/A
Total	161	25	

5 Table 2 – Summary of transformation experiments. 234, RN, TAB represent
     different sources of the eIF4E gene. AS = antisense, S = sense.
     NPTII result confirms transformation. Northern blotting reflects
     degree of expression of the eIF4E mRNA. H signifies a high level
     of expression was detected; M = moderate level of eIF4E mRNA
10   expression and L = low/undetectable expression.

Transgenic line	NPT2 PCR	Northern Blotting	TEV resistance
234-AS1	positive	H	
234-AS2	positive	H	
234-AS3	positive	M	
234-AS4	positive	H	
234-AS6	positive	M	
234-AS8	positive	H	
234-AS9	positive	H	
234-AS10	positive	H	
234-AS11	positive	M	
234-AS12	positive	H	
234-AS13	positive	L	resistant segregates
234-AS14	positive	H	
234-AS15	positive	H	
234-AS16	positive	L	resistant segregates
234-AS17	positive	H	
234-AS18	positive	M	
234-AS19	positive	L	
234-AS20	positive	H	
234-AS22	positive	H	
234-AS23	positive	H	
234-AS24	positive	M	
234-AS25	positive	H	
234-AS26	positive	M	
234-AS27	positive	L	resistant segregates
234-AS28	positive	H	
234-AS29	positive	H	
234-AS30	positive	L	
234-AS31	positive	H	

234-AS32	positive	L	resistant segregates
234-AS33	positive	L	
234-AS34	positive	H	
234-AS35	positive	H	
234-AS36	positive	L	
234-AS37	positive	L	
234-AS38	positive	L	
234-AS40	positive	H	
234-AS42	positive	H	
RN-AS1	positive	H	
RN-AS2	positive	H	
RN-AS3	positive	H	
RN-AS4	positive	H	
RN-AS5	positive	H	
RN-AS8	positive	H	
RN-AS9	positive	H	
RN-AS10	positive	H	
RN-AS11	positive	H	
RN-AS13	positive	L	resistant segregates
RN-AS14	positive	H	
RN-AS15	positive	H	
RN-AS16	positive	H	
RN-AS17	positive	H	
RN-AS18	positive	H	
RN-AS19	positive	H	
RN-AS20	positive	L	resistant segregates
RN-AS21	positive	H	
RN-AS22	positive	H	
RN-AS23	positive	H	
RN-AS24	positive	H	
RN-AS25	positive	H	
RN-AS26	positive	H	
RN-AS27	positive	M	
RN-AS28	positive	H	
RN-AS29	positive	H	
RN-AS30	positive	H	
RN-AS31	positive	M	
RN-AS32	positive	H	
RN-AS33	positive	H	
RN-AS34	positive	H	
RN-AS35	positive	H	
RN-AS36	positive	M	
RN-AS37	positive	H	
RN-AS38	positive	L	
RN-AS39	positive	H	
RN-AS40	positive	H	
RN-AS41	positive	H	
TAB-AS1	positive	H	
TAB-AS2	positive	H	

TAB-AS4	positive	H	
TAB-AS5	positive	L	resistant segregates
TAB-AS7	positive	H	
TAB-AS8	positive	H	
TAB-AS10	positive	L	resistant segregates
TAB-AS11	positive	H	
TAB-AS12	positive	H	
TAB-AS14	positive	M	
TAB-AS15	positive	H	
TAB-AS16	positive	M	
TAB-AS17	positive	M	
TAB-AS19	positive	M	
TAB-AS20	positive	H	
TAB-AS21	positive	H	
TAB-AS22	positive	L	
TAB-AS23	positive	H	
TAB-AS24	positive	L	resistant segregates
TAB-AS25	positive	H	
TAB-AS26	positive	M	
TAB-AS27	positive	L	
TAB-AS28	positive	H	
TAB-AS29	positive	H	
TAB-AS30	positive	H	
TAB-AS31	positive	H	
TAB-AS32	positive	L	resistant segregates
TAB-AS33	positive	H	
TAB-AS34	positive	H	
TAB-AS35	positive	M	
TAB-AS36	positive	H	
234-S1	positive	L	
234-S2	positive	H	
234-S3	positive	M	
234-S4	positive	L	
234-S5	positive	M	
234-S6	positive	H	
234-S7	positive	L	
234-S8	positive	M	
234-S9	positive	H	
234-S10	positive	H	
234-S11	positive	M	
234-S12	positive	H	
234-S13	positive	H	
RN-S1	positive	H	
RN-S2	positive	H	
RN-S3	positive	L	
RN-S4	positive	H	
RN-S5	positive	L	
RN-S7	positive	M	
RN-S8	positive	H	

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RN-S9	positive	M	
RN-S10	positive	M	
RN-S11	positive	H	
RN-S12	positive	M	
RN-S13	positive	H	resistant segregates
RN-S18	positive	L	
RN-S19	positive	H	
TAB-S1	positive	H	
TAB-S2	positive	H	
TAB-S4	positive	H	
TAB-S7	positive	M	
TAB-S8	positive	H	
TAB-S9	positive	H	
TAB-S10	positive	H	
TAB-S11	positive	L	

Example 15 – Mutations In Translation Initiation Factor Eif4e That Confer Resistance To Potyvirus Infection In *Capsicum* Abolish Interaction With *Tobacco Etch Virus* Vpg In A Non-Specific Manner

[0134]   The objectives of the study were to identify and characterize the host factor responsible for *pvr1*-mediated resistance in pepper, and to establish the relationship of *pvr1* to *pvr2²* with respect to their common resistance to TEV-HAT. Based on the prevalent role of NIa in determining potyvirus replication and movement functions with respect to recessive resistance genes and the well-documented interaction of NIa with host cap-binding factors, eIF4E and eIF(iso)4E were examined as candidate genes for *pvr1*.

[0135]   In plants, the mRNA cap-binding protein eIF(iso)4E and the scaffolding protein eIF(iso)4G interact to form the core of the eIF(iso)4F complex, which supports translation initiation predominantly from mRNAs with unstructured 5′ leaders (Gallie et al., "eIF4G Functionally Differs from eIFiso4G in Promoting Internal Initiation, Cap-Independent Translation, and Translation of Structured mRNAs," *J. Biol. Chem.* 276:36951-36960 (2001)). In contrast, eIF4F, comprised of the mRNA cap-binding protein eIF4E and scaffolding protein eIF4G, supports translation initiation of mRNAs with more complex 5′ structures and may promote translation under cellular conditions that inhibit cap-dependent translation such as viral infection. In *Arabidopsis,* eIF4E and eIF(iso)4E share

52% identity at the amino acid level and show different patterns of expression (Rodriguez et al., "The *Arabidopsis thaliana* cDNAs Coding for eIF4E and eIF(iso)4E are Not Functionally Equivalent for Yeast Complementation and are Differentially Expressed During Plant Development," *Plant J.* 13:465-473

5   (1998)), although both are strongly expressed in young tissues that support high levels of potyviral replication.

[0136]   The recessive *pvr1* gene from *Capsicum chinense* confers broad-spectrum resistance to all known strains of *Potato virus Y* (PVY), *Pepper mottle virus* (PepMoV), and most known TEV strains (Kyle et al., "Proposed Revision of

10   Nomenclature for Potyvirus Resistance Genes in *Capsicum*," *Euphytica* 97:183-188 (1997)). Broad-spectrum resistance to several PVY and TEV strains, but not PepMoV, is also conferred by the recessive *Capsicum annuum* gene *pvr2²*. These genes have a common mechanism of resistance (blocked replication) to the highly-aphid transmissible (HAT) strain of TEV (Deom et al., "Resistance to

15   Tobacco Etch Virus in *Capsicum annuum*: Inhibition of Virus RNA Accumulation," *Mol. Plant Microbe Interact.* 10:917-921 (1997); and Murphy et al., "Genetic Mapping of the pvr1 Locus in *Capsicum spp.* and Evidence that Distinct Potyvirus Resistance Loci Control Responses that Differ at the Whole Plant and Cellular Levels," *Mol. Plant Microbe Interact.* 11:943-951 (1998)). In

20   addition to a shared mechanism of resistance to at least one viral isolate, these broad-spectrum resistance genes share a common position on comparative genetic maps, raising the question of their genetic relationship to one another. Another allele at the *pvr2* locus, *pvr2¹*, provides narrow-spectrum resistance to only pathotype 0 of PVY, apparently by blocking cell-to-cell movement (Arroyo et al.,

25   "Impaired Cell-to-Cell Movement of Potato Virus Y in Pepper Plants Carrying the Y-A (pr2-1) Resistance Gene," *Mol. Plant Microbe Interact.* 9:314-318 (1996)).

[0137]   Several recent reports have confirmed that both eIF(iso)4E and eIF4E play a critical role in potyviral infection. Map-based cloning in *Arabidopsis* revealed that induced nonsense mutations in eIF(iso)4E resulted in

30   broad-spectrum potyvirus resistance to TEV and TuMV (Lellis et al., "Loss-of-Susceptibility Mutants of *Arabidopsis thaliana* Reveal an Essential Role for eIF(iso)4E During Potyvirus Infection," *Curr. Biol.* 12:1046-1051 (2002), which is hereby incorporated by reference in its entirety). A transposon-disrupted

eIF(iso)4E mutant identified through reverse genetics has since been shown to confer broad-spectrum resistance to TuMV and *Lettuce mosaic virus* (LMV) (Duprat et al., "The Arabidopsis Eukaryotic Initiation Factor (iso)4E is Dispensable for Plant Growth but Required for Susceptibility to Potyviruses,"

5   *Plant J.* 32:927-934 (2002), which is hereby incorporated by reference in its entirety). Further evidence from *Capsicum* and lettuce demonstrates that naturally occurring mutations in eIF4E are responsible for strain-specific PVY resistance conferred by *pvr2*[1] and *pvr2*[2] in pepper (Ruffel et al., "A Natural Recessive Resistance Gene Against Potato Virus Y in Pepper Corresponds to the Eukaryotic

10   Initiation Factor 4E (eIF4E)," *Plant J.* 32:1067-1075 (2002), which is hereby incorporated by reference in its entirety) and for resistance to LMV conferred by *mo1* (Nicaise et al., "The Eukaryotic Translation Initiation Factor 4E Controls Lettuce Susceptiblity to the Potyvirus Lettuce Mosaic Virus," *Plant Phys.* 132:1272-1282 (2003), which is hereby incorporated by reference in its entirety).

15   [0138]   Translation initiation factors eIF4E and eIF(iso)4E have been shown to interact with the multifunctional potyvirus protein NIa and to be important host factors for potyviral infection. It has been shown that mutations in the eIF4E homolog eIF4E-687 result in broad-spectrum potyvirus resistance conferred by *pvr1* in *Capsicum*, a gene widely deployed in agriculture. Two other

20   resistance genes with narrower resistance spectra, *pvr2*[1] and *pvr2*[2], were determined to be alleles at the *pvr1* locus and were re-designated *pvr1*[1] and *pvr1*[2]. Mutations in *pvr1*, *pvr1*[1] and *pvr1*[2] grouped to similar regions of the gene and were predicted by protein homology models to cause conformational shifts in the encoded proteins. Because the interaction between eIF(iso)4E and NIa is

25   considered critical for potyvirus infection, yeast two-hybrid and GST pull-down assays were performed with alleles of eIF4E-687 and NIa or VPg, respectively, from two different strains of *Tobacco etch virus* (TEV) that differentially infected *Capsicum* lines carrying these genes. The eIF4E homolog eIF4E-687 isolated from *pvr1*, *pvr1*[1] and *pvr1*[2] genotypes failed to bind NIa/VPg from either strain of

30   TEV, however, this failure to interact did not consistently correlate with infectivity. These data suggest that interruption of the interaction between NIa/VPg and eIF4E-687 may be necessary but is not sufficient for potyvirus resistance *in vivo*. Among the three resistance alleles, only the *pvr1* gene product

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also failed to bind m^7-GTP cap-analog columns, suggesting that disrupted cap-binding is not required for potyvirus resistance.

[0139]   The results of this study both complement and extend these findings by demonstrating that mutations in pepper eIF4E (eIF4E-687) are also responsible for broad-spectrum potyvirus resistance conferred by the *pvr1* allele in *Capsicum*, and the *pvr2* alleles are actually variants at the *pvr1* locus. The results show that *pvr1* carries a unique set of amino acid substitutions relative to $pvr2^1$ and $pvr2^2$. Described is an additional amino acid substitution in the $pvr2^2$ allele, not previously reported, confirmed by sequencing eIF4E-687 from three $pvr2^2$ / $pvr2^2$ pepper cultivars. When recombinant $pvr1^+$, *pvr2*, $pvr2^1$, and $pvr1^2$ eIF4E proteins were assayed for their ability to bind m^7-GTP cap analog columns, only *pvr1* failed to bind indicating that disruption of this function may not be necessary for resistance and that knockout mutations at this locus may be viable. To test the hypothesis proposed in earlier studies that amino acid substitutions in the $pvr2^1$ and $pvr2^2$ alleles disrupted interaction with TEV-HAT NIa resulting in resistance, a systematic study was conducted of eIF4E-687/NIa interactions *in vitro*. In every case, the mutations in the *pvr1*, $pvr2^1$ and $pvr2^2$ alleles resulted in disrupted eIF4E-687 binding of NIa from either TEV strain; however, the lack of interaction was not correlated with resistance phenotypes. These findings require revision of gene nomenclature at this locus and indicate that potyvirus resistance cannot be predicted solely on the basis of NIa/VPg binding or cap-binding with eIF4E-687 proteins. It is likely that the determinants of specificity in this system will reside elsewhere.

Example 16 – Plants, Viruses and Populations for Genetic Studies

[0140]   Seeds of *C. annuum* 'NuMex RNaky' (RN), 'Early Cal Wonder' (ECW), 'Florida VR2' (VR2), 'Yolo Y' (YY), 'Del Ray Bell' (DRB), and breeding lines '3618', '5502', and '4074' were obtained from Asgrow Seed Co., San Juan Bautista, CA. *C. annuum* 'Dempsey' (DEMP) was provided by M. Deom, University of Georgia and *C. annuum* 'Jupiter' was provided by Syngenta Seeds, Naples, FL; *C. chinense* PI 152225, PI 159234 (234), and PI 159236 were obtained from the USDA Southern Regional Plant Introduction Station,

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Experiment, GA; *C. chinense* 'Habanero' (Hab) was obtained from Tomato
Growers Supply Co., Fort Myers, FL. TEV-HAT and TEV-NW (non-wilting)
cultures were obtained from T. Pirone, University of Kentucky, Lexington, KY.
PepMoV-FL (Florida) was obtained from T. Zitter, Cornell University, Ithaca,

5   NY. All potyvirus strains were maintained on TMV-resistant *Nicotiana tabacum*
'Kentucky 14' and were transferred every 4-8 weeks.

[0141]   For genetic complementation experiments, the set of resistant
parents (DEMP, VR2, 3628, 5502) were intercrossed in all combinations and
crossed with the susceptible parents Jupiter and ECW. Parents and F_1 progenies

10   were inoculated with TEV-HAT and assayed for symptoms and presence of viral
antigen by indirect ELISA.

[0142]   DNA samples from F_2 individuals of an interspecific mapping
population (AC population) used in a previous study to map the *pvr1* locus were
used to assess genetic markers for the *pvr1* locus (Livingstone et al., "Genome

15   Mapping in *Capsicum* and the Evolution of Genome Structure in the Solanaceae,"
Genetics 152:1183-1202 (1999), which is hereby incorporated by reference in its
entirety). A second population of 114 F_3 families was generated for this study
from an intraspecific cross between *C. chinense* 'Habanero' and *C. chinense* PI
159234 (CC population). This population was scored for resistance to TEV-HAT

20   and PepMoV in two separate screens. DNA samples were extracted as described
previously for genotypic analysis (Livingstone et al., "Genome Mapping in
Capsicum and the Evolution of Genome Structure in the Solanaceae," *Genetics*
152:1183-1202 (1999); and Murphy et al., "Genetic Mapping of the pvr1 Locus in
Capsicum spp. and Evidence that Distinct Potyvirus Resistance Loci Control

25   Responses that Differ at the Whole Plant and Cellular Levels," *Mol. Plant
Microbe Interact.* 11:943-951 (1998), which are hereby incorporated by reference
in their entirety).

Example 17 – **Virus Screening Procedure**

30   [0143]   The first two leaves of greenhouse grown seedlings at the 4-6 leaf
stage were inoculated using carborundum with plant sap from TEV-HAT-infected
or PepMoV-infected tobacco plants ground in ice-cold 0.05 M potassium

phosphate buffer, pH 8. Systemic infection was assayed at 21 days post-inoculation (dpi) by visual symptoms and confirmed by indirect ELISA (Voller et al., "The Detection of Viruses by Enzyme-Linked Immuno-Sorbent Assay," *J. Gen. Virol.* 33:165-167 (1976), which is hereby incorporated by reference in its
5   entirety).

Example 18 – RNA Isolation, RNA Blots and RT-PCR

[0144]   Total RNA was isolated from young pepper leaves using Trizol reagant (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.
10   RNA blots were done according to standard methods (Sambrook et al., "Molecular Cloning : A Laboratory Manual," Third Edition, Cold Spring Harbor, NY:Cold Spring Harbor Laboratory Press (2001), which is hereby incorporated by reference in its entirety). First strand cDNA was synthesized in 25 µl containing 2µg total RNA and 500 ng oligo dT using M-MLV reverse transcriptase
15   (Promega, Madison, WI) according to the manufacturer's instructions. For RT-PCR, 2 µl of cDNA was added to a reaction volume of 25 µl containing 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.2 mM each dNTP, 0.4 uM each forward and reverse primer, and 1 unit *Taq* polymerase (New England Biolabs, Beverly, MA). PCR cycling conditions were
20   95°C 3 m, (95°C 30 s, 55°C 30 s, 72°C 5 m) x 1, (95°C 30 s, 55°C 30 s, 72°C 90 s) x 29, 72°C 10 min. Full-length eIF4E-687 ORF primers (forward 5'-ATGGCAACAGCTGAAATGG-3' (SEQ ID NO:9); reverse 5'-TATACGGTGTAACGATTCTTGGCA-3' (SEQ ID NO:10) were based on tomato eIF4E sequence (Genbank accession AF259801). Full-length eIF(iso)4E
25   ORF primers (forward 5'-AACAATGGCCACCGAAGC-3' (SEQ ID NO:15); reverse 5'-ATTTCACAGTATATCGGCTCT-3' (SEQ ID NO:16)) were based on published tomato sequence (TIGR accession TC103222) (www.tigr.org). Full-length eIF4E-537 ORF primers (forward 5'-TTAGGCAAACCAATCACAATG-3' (SEQ ID NO:19); reverse 5'-CCTGTTGTAACGATAGAACTA-3' (SEQ ID
30   NO:20)) were based on published tomato sequence (TIGR accession TC96888). PCR products were run on 1.5% agarose. Gel slices were purified using the

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Qiaquick gel purification kit (Qiagen, Valencia, CA) and cloned using the pGEM-
T Easy kit (Promega, Madison, WI).

Example 19 – cDNA Sequence Alignment and Protein Modeling

[0145]   At least two positive clones were sequenced from both ends for
each PCR product and analyzed using Seqman software (DNASTAR Inc.,
Madison, WI). Amino acid sequence alignments were produced using the Clustal
algorithm within Megalign software (DNASTAR). For protein homology models,
pepper sequence was submitted to the SwissProt database via the application
DeepView (www.expasy.ch/spdbv/text/server.htm). The model was generated
using the murine crystal structure for eIF4E (sequence 1EJ1.B).

Example 20 – Genome-Walking and Alignment of Genomic Sequence

[0146]   For genome-walking 5' to eIF4E-687, nested internal primers 79R
5'-CACCTTCTTCAACTTCATCATCTGCCT-3' (SEQ ID NO:21) and 56R 5'-
CATCATCTGCCTTCATTAGCATTCAAT-3' (SEQ ID NO:14) were designed
for use with the nested primers included in the Universal Genome-Walking Kit
(Clontech, Palo Alto, CA). Subsequent PCR reactions followed manufacturer's
instructions. Genome-walking was also used to span the 3.6 kb intron I of eIF4E-
687. Nested primer sequences were 9F 5'-
AGCTGAAATGGAGAAAACGACGA-3' (SEQ ID NO:22) and 39F 5'-
TTTGATGAAGCTGAGAAGGTGAAATTG-3' (SEQ ID NO:23) for the first
walk and MW1F 5'-CGGCTTGAAAGTTCAGTTAGTCAAC-3' (SEQ ID
NO:24) and MW2F 5'-GCCAACTAACTTAGCTACTAAGATTTTCAG-3' (SEQ
ID NO:25) for the second walk. The remainder of intron I was spanned using
primers I1F 5'-GCCATTCGACTAATCCTCAGCAAC-3' (SEQ ID NO:26) and
321WR 5'-CCTCCATTGGCACATACAGGATCT-3' (SEQ ID NO:27). Intron II
was spanned with the E23 marker using primers E23F 5'-
AATATCCATCACCCAAG-3' (SEQ ID NO:11) and E23R 5'-
GCTCCACATATTTCATC-3' (SEQ ID NO:12). Intron III was spanned using
primer I2F 5'-CACTCTAGTAATATTATTTTCTGT-3' (SEQ ID NO:28) and

primer 625R 5'-TGAAATATGAAGCCAACACTG-3' (SEQ ID NO:29). For genome-walking through intron IV and the 3' end of the gene, nested internal primers 580F 5'-GGTAAGCAATGGAAGCAGTTTCTG-3' (SEQ ID NO:30) and 595F 5'-CAGTTTCTGGATTACAGCGACAGT-3' (SEQ ID NO:31) were

5   utilized. Bands were gel-purified from agarose using the Qiaquick gel extraction kit, cloned into pGEM-T Easy, and sequenced. A contig was created that spanned the eIF4E locus using overlapping PCR clones with Seqman software (DNASTAR).

10   **Example 21 – DNA Blots**

[0147]   DNA blotting and hybridization were done as reported previously (Livingstone et al., "Genome Mapping in *Capsicum* and the Evolution of Genome Structure in the Solanaceae," *Genetics* 152:1183-1202 (1999), which is hereby incorporated by reference in its entirety). Probes were amplified via PCR from

15   cloned DNA fragments, purified from agarose using the Qiaquick gel extraction kit and labeled with ^{32}P using the Prime-It Random Primer Labeling Kit (Promega) according to manufacturer's instructions. Labeled probe was purified through a Sephadex G50 column, incubated with blots overnight at 65°C, then washed once with 2X SSC, once with 1X SSC and twice with 0.5X SSC. All

20   washes included 0.1% SDS. Filters were placed on Kodak XAR-5 film. Genetic maps were assembled using MapMaker /EXP v3.0b (Livingstone et al., "Genome Mapping in *Capsicum* and the Evolution of Genome Structure in the Solanaceae," *Genetics* 152:1183-1202 (1999), which is hereby incorporated by reference in its entirety).

25

Example 22 – CAPS Markers

[0148]   The upstream primer used to amplify the promoter region containing the *Ssp*I CAPS differential cleavage site was based on sequence obtained by genome-walking. The reverse primer lies 56 bases within the eIF4E-

30   687 open reading frame. The reaction volume of 50 μl contained 50 ng genomic DNA, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.2 mM each dNTP, 0.4 μM each forward primer 5'-

TTACACGCGCCGATACACTTG-3' (SEQ ID NO:13) and reverse primer 5'-

CATCATCTGCCTTCATTAGCATTCAAT-3' (SEQ ID NO:14), and 2 units *Taq*

polymerase (New England Biolabs). Cycling conditions were 95°C 3 m, (95°C 30

s, 60°C 30 s, 72°C 5 m) x 1 cycle, (95°C 30 s, 60°C 30 s, 72°C 90 s) x 29 cycles,

5   72°C 10 m. A 20 μl aliquot of PCR product was digested with 2.5 units of *Ssp*I

(New England Biolabs) in a 30 μl reaction volume.

Example 23 – Agro-Infiltration Transient Assay for Functional Complementation

10   **[0149]**   For transient expression of eIF4E via agroinfiltration, eIF4E-234

and eIF4E-RN were cloned into the pBI121 plasmid. *Agrobacterium tumefaciens*

LBA4404 was transformed by electroporation with the constructs above and the

empty vector and grown overnight at 28°C in LB medium containing 50 μg/ml

kanamaycin and 50 μg/ml streptomycin. The overnight cultures were washed,

15   resuspended in induction medium and induced overnight. Cells were collected by

centrifugation and resuspended in 10 mM MES and 10 mM $MgCl_2$ containing 150

μM acetosyringone to an OD_{600} of 0.2, then infiltrated using a 1 ml syringe into

the first true leaves of susceptible (*C. chinense* Habanero) or resistant (*C. chinense*

234) pepper plants. Infiltrated leaves were then inoculated with TEV-HAT 3 days

20   after infiltration. Uninoculated and mock-inoculated controls were also included.

Five days after TEV inoculation, leaves were collected and TEV accumulation

was test by immuno-blotting using a anti-TEV coat protein antisera. eIF4E

expression was also assayed by the same method using human anti-eIF4E

antibody (New England Biolabs).

25

Example 24 — Yeast Two-Hybrid Analysis

[0150]   Yeast two-hybrid analysis was performed according to published

methods (Golemis et al., "Interaction Trap/Two-Hybrid System to Identify

Interacting Proteins," In Ausubel et al., eds., *Current Protocols in Molecular*

30   *Biology*, New York:Wiley, pp. 20.1.1-20.1.28 (1996), which is hereby

incorporated by reference in its entirety). Yeast strains and plasmid vectors were

provided by G. Martin (Boyce Thompson Institute, Ithaca, NY). A bait plasmid

pEG202 was used for the fusion of NIa to the DNA binding domain of LexA. The DNA sequences encoding NIa from TEV-HAT and NIa from TEV-NW were amplified by PCR from full-length clones provided by J. Murphy (Auburn University, Auburn, AL) using forward primer 5'-

5   GCC<u>GAATTC</u>ATGGGGAAGAAGAATCAGA-3' (SEQ ID NO:32) (*Eco*RI site underlined) and reverse primer 5'- CC<u>CTCGAG</u>CTATTGCGTGTACACCAATTC-3' (SEQ ID NO:33) (*Xho*I site underlined). A prey plasmid pJG4-5 was used to express *Capsicum* eIF4E-687 alleles. The eIF4E-687 sequences from different pepper genotypes known to be

10   homozygous for each resistance allele were amplified using forward primer 5'- <u>GAATTC</u>ATGGCAACAGCTGAA-3' (SEQ ID NO:34) (*Eco*RI site underlined) and 5'-<u>CTCGAG</u>CTATACGGTGTAACG-3' (SEQ ID NO:35) (*Xho*II site underlined). The amplified fragments were digested with *Eco*RI and *Xho*I and cloned into corresponding restriction sites of pEG202 and pJG4-5, respectively.

15   NIb of TEV-HAT was amplified by PCR using forward primer 5'- CC<u>GAATTC</u>ATGGGAGAGAAGAGGAAATG-3' (SEQ ID NO:36) (*Eco*RI site underlined) and reverse primer 5'- CC<u>CTCGAG</u>CTACTGAAAATAAAGATTCTC-3' (SEQ ID NO:37) (*Xho*I site underlined) and cloned into pEG202 as a positive control for interaction with NIa

20   in the β-galactosidase assay. All constructs were confirmed by sequencing. Bait recombinant plasmids were transformed into yeast strain EGY48 containing the *lacZ* reporter plasmid pSH18-34 using the lithium acetate method. Yeast cells containing pSH18-34 and bait plasmids were subsequently transformed with the prey plasmid pJG4-5:NIa. For two-hybrid assays, transformants were grown in

25   complete minimal medium lacking histidine, tryptophan, and uracil, supplemented with 2% galactose/1% raffinose and X-gal. The degree of interaction was determined by the β-galactosidase liquid assay (Victoria, "Yeast Vectors and Assays for Expression of Cloned Genes, In Ausubel et al., eds., *Current Protocols in Molecular Biology*, New York:Wiley, pp. 13.6.1-13.6.4 (1996), which is hereby

30   incorporated by reference in its entirety) and protein expression was assayed by immmunoblots probed with anti-eIF4E to assure equivalent expression and stability in all treatments.

Example 25 – GST *In Vitro* Pull-Down Assay

[0151]   Each of the three resistant alleles, eIF4E-234 (*pvr1*), eIF4E –YY (*pvr2¹*), eIF4E –DEMP (*pvr2²*), and the susceptible allele, eIF4E –RN (*pvr1⁺*), were cloned into the pET16b vector and translated *in vitro* using TnT coupled reticulocyte lysate (Promega). GST, GST-VPgHAT, and GST-VPgNW proteins were bound to 10 µl GST-Sepharose beads and resuspended in 195 µl IPAB-gelatin buffer (20 mM HEPES pH7.8, 150 mM KCl, 0.1% gelatin, 0.1% Triton X-100, 0.1% NP-40, 5 mM MgCl₂, 2 mM DTT). TnT translation products (5 µl) were added to prepared GST fusion Sepharose, mixed by end-over rotation at 4°C for overnight, and washed 3x with 1 X PBS buffer containing 2 mM DTT. The Sepharose beads were resuspended in 20 µl SDS-PAGE loading buffer and incubated for 2 min at 100°C. Released proteins were separated by SDS-PAGE. Following electrophoresis, gels were fixed, dried, and exposed to a phosphorimaging screen.

Example 26 – Cap-Binding Assays

[0152]   The coding region of pepper eIF4E-687 was amplified by PCR with forward primer 5'- CCATATGGCAACAGCTGA-3' (SEQ ID NO:38) (*Nde*I site underlined) and reverse primer 5'- CCCTCGAGCTATACGGTGTAACGA-3' (SEQ ID NO:39) (*Xho*I site underlined). The amplified fragments were then digested by *Nde*I and *Xho*I and cloned into pET16b (Novagen, Madison, WI) in frame with the 6 X histidine tag. The constructs were confirmed by sequencing before transformation into *E. coli* BL21(DE3)pLysS (Novagen). Expression of the recombinant proteins and purification of the proteins by m⁷GTP-Sepharose 4.B (Amersham Bioscience, Piscataway, NJ) were carried out as described previously with minor modification (Hagedorn et al., "Expression of Functional eIF-4E Human: Purification, Detailed Characterization, and Its Use in Isolating eIF-4E Binding Proteins," *Protein Expr. Purif.* 9:53-60 (1997), which is hereby incorporated by reference in its entirety). Expression of recombinant proteins was induced at 20°C for 20h by addition of 20 µM IPTG. The bacterial cells from 500 ml culture were resuspended in 5 ml of extraction buffer (20 mM Tris-HCl, pH

7.5 (4°C), 100 mM KCl, 1 mM EDTA, 1 mM DTT, 1mM PMSF) and sonicated.
After cell lysis, Triton X-100 was added to a final concentration of 0.1% and
stirred for 15 m at 4°C. The samples were centrifuged 20 m at 22,000 x g; the
supernatant was recovered for the cap-binding assay and applied to a 0.5 ml

5   affinity column saturated with extraction buffer. The column was washed
extensively with extraction buffer and eIF4E-687 protein was eluted using
extraction buffer containing 100 uM m⁷GTP. SDS-PAGE and immunoblot
analysis were carried out as described previously (Murphy et al., "Isolation and
Viral Infection of *Capsicum* Leaf Protoplasts," *Plant Cell Rep.* 13:397-400

10   (1994), which is hereby incorporated by reference in its entirety), using human
anti-eIF4E antibody (New England Biolabs) for detection of recombinant eIF4E-
687.

Example 27 – Cosegregation of eIF4E-687 with *pvr1*

15   [0153]   Primers based on a 696 bp tomato eIF4E coding sequence
(Genbank accession AF259801) were used to amplify a 687 bp pepper eIF4E
fragment (eIF4E-687) with 87% nucleotide sequence identity to tomato and 62%
nucleotide sequence identity to *Arabidopsis* eIF4E (EMBL accession Y10548). A
hybridization probe generated from eIF4E-687 identified a polymorphism that

20   mapped precisely to *pvr1* in the interspecific AC mapping population (FIG. 8A)
(Murphy et al., "Genetic Mapping of the pvr1 Locus in *Capsicum spp.* and
Evidence that Distinct Potyvirus Resistance Loci Control Responses that Differ at
the Whole Plant and Cellular Levels," *Mol. Plant Microbe Interact.* 11:943-951
(1998), which is hereby incorporated by reference in its entirety). The *Ssp*I CAPS

25   marker based in the 5' upstream region of the gene confirmed this result in the
intra-specific CC population (FIG. 8B).
[0154]   Primers based on a 603 bp tomato eIF(iso)4E sequence (TIGR
accession TC103222) amplified a 609 bp pepper eIF(iso)4E with 89% nucleotide
sequence identity to tomato and 63% nucleotide sequence identity to *Arabidopsis*

30   eIF(iso)4E (EMBL accession Y10547). DNA blot hybridization of pepper
eIF(iso)4E in the AC population showed that it was unlinked to *pvr1*, localizing
instead to a chromosomal interval containing the recessive resistance gene *pvr6*,

reported to confer resistance to the potyvirus *Pepper veinal mottle virus* (PVMV) when combined with *pvr2²* (Caranta et al., "A Complementation of Two Genes Originating from Susceptible *Capsicum annuum* Lines Confers a New and Complete Resistance to Pepper Veinal Mottle Virus," *Phytopathology* 86:739-743

5   (1996), which is hereby incorporated by reference in its entirety).

[0155]   Primers based on the ORF of a 537 bp tomato eIF4E homolog (TIGR accession TC96888) amplified a 537 bp pepper fragment with 96% nucleotide sequence identity to tomato and 71% nucleotide sequence identity to *Arabidopsis* eIF4E. This eIF4E homolog (eIF4E-537) did not map near known

10 resistance genes and lacked any characteristic polymorphisms between resistant and susceptible genotypes. Tryptophan and other cap-binding residues (Marcotrigiano et al., "Cocrystal Structure of the Messenger RNA 5' Cap-Binding Protein (eIF4E) Bound to 7-Methyl-GDP," *Cell* 89:951-961 (1997), which is hereby incorporated by reference in its entirety) were highly conserved among all

15 eIF4E isoforms and homologs, consistent with functional redundancy for cap-binding within the pepper genome.

Example 28 – Genetic Complementation Analysis of *pvr1* and *pvr2²* Demonstrates Allelism

20 [0156]   Allelism between *pvr2¹* and *pvr2²* was established in early studies using PVY pathotype 0 (Cook, "A Mutation for Resistance to Potato Virus Y in Pepper.," *Phytopathology* 51:550-552 (1961), which is hereby incorporated by reference in its entirety). The formal genetic relationship of *pvr1* and *pvr2²* was never addressed. Originally described as alleles of the *et* locus for resistance to

25 TEV (Deom et al., "Resistance to Tobacco Etch Virus in *Capsicum annuum*: Inhibition of Virus RNA Accumulation," *Mol. Plant Microbe Interact.* 10:917-921 (1997); and Herold et al., "Poliovirus RNA Replication Requires Genome Circularization Through a Protein-Protein Bridge," *Mol. Cell* 7:581-591 (2001), which are hereby incorporated by reference in their entirety), they were

30 subsequently assigned to distinct loci (Sachs et al., "Starting at the Beginning, Middle, and End: Translation Initiation in Eukaryotes," *Cell* 89:831-838 (1997), which is hereby incorporated by reference in its entirety). Comparative mapping data places both genes near a common RFLP marker TG135, which in tomato is

closely linked to *pot-1*, a recessive resistance gene in tomato for TEV and PVY resistance (Murphy et al., "Genetic Mapping of the pvr1 Locus in *Capsicum spp.* and Evidence that Distinct Potyvirus Resistance Loci Control Responses that Differ at the Whole Plant and Cellular Levels," *Mol. Plant Microbe Interact.*

5   11:943-951 (1998); Parella et al., "Recessive Resistance Genes Against Potyviruses are Localized in Colinear Genomic Regions of the Tomato (Lysopersicon spp.) and Pepper (*Capsicum spp.*) Genomes," *Theor. Appl. Genet.* 105:855-861 (2002), which are hereby incorporated by reference in their entirety). Both *pvr1* and *pvr2²* confer resistance to TEV-HAT infection at the cellular level

10   (Deom et al., "Resistance to Tobacco Etch Virus in *Capsicum annuum*: Inhibition of Virus RNA Accumulation," *Mol. Plant Microbe Interact.* 10:917-921 (1997); Murphy et al., "Genetic Mapping of the pvr1 Locus in *Capsicum spp.* and Evidence that Distinct Potyvirus Resistance Loci Control Responses that Differ at the Whole Plant and Cellular Levels," *Mol. Plant Microbe Interact.* 11:943-951

15   (1998), which are hereby incorporated by reference in their entirety), therefore this strain can be used to evaluate genetic complementation based on the resistance phenotypes of F_1 progeny derived from the two resistant sources. When genetic complementation for resistance to TEV-HAT was assessed, *pvr1* and *pvr2²* failed to complement (FIG. 9A). Because the *pvr1* allele was described

20   first (Greenleaf, "Inheritance of Resistance to Tobacco Etch Virus in *Capsicum frutescens* and in *Capsicum annuum*," *Phytopathology* 46:371-375 (1956), which is hereby incorporated by reference in its entirety), the designation *pvr1* should take priority for the eIF4E-687 locus. Based upon genetic complementation results and rules for potyviral resistance gene nomenclature in *Capsicum*, the

25   *pvr2¹* allele should be redesignated *pvr1¹*, while the *pvr2²* allele should be redesignated *pvr1²*.

Example 29 – Functional Complementation of a Resistant Genotype with eIF4E

30   [0157]   Functional complementation for susceptibility was established via transient expression of resistant (eIF4E-234, *pvr1*) and susceptible (eIF4E-RN, *pvr1⁺*) alleles via agroinfiltration into susceptible (Hab) and resistant (234) seedlings, together with appropriate controls for infiltration. Infiltrated and

control seedlings were challenged with TEV-HAT and assayed by immunoblot for expression of eIF4E and TEV CP. Mock-inoculated and uninoculated controls were also included. TEV CP antigen was only detected in the positive control, the susceptible genotype inoculated with TEV, and in 234 plants infiltrated with the

5   construct expressing the susceptible allele (FIG. 9B).

Example 30 – The Genetic Structure of the *Capsicum* eIF4E-687 Locus is Similar to *Arabidopsis* eIF4E

[0158]   Genomic sequence was obtained by genome-walking and genomic

10 PCR to confirm the full-length gene sequence of eIF4E-687 and to describe the molecular structure of the locus (FIG. 10). A 66 bp region with 92% nucleotide sequence identity to bases 80-146 of the eIF4E-687 ORF was found approximately 1.3 kb upstream of the gene. Primers based 1.1 kb upstream of the gene and in exon 1 were used to generate the *Ssp*I P56 CAPS marker shown in

15 FIG. 8B. The length of the first exon beginning from the ATG start codon was 278 bp. The first intron was quite large, measuring 3.6 kb. The PCR marker E23, generated using primers anchored in exons 2 (166 bp) and 3 (126 bp), spanned the 110 bp second intron and generated a *Mse*I polymorphism that also cosegregated with *pvr1* resistance. Intron III was 1.1 kb, while exon 4 was 66 bp. A final

20 intron (intron IV) was 82 bp. Exon 5 up to the stop codon TAA was a total of 51 bp. Sequence corresponding to 5' untranslated region (28 bp) and 3' untranslated region (156 bp) was consistent with that previously reported (Ruffel et al., "A Natural Recessive Resistance Gene Against Potato Virus Y in Pepper Corresponds to the Eukaryotic Initiation Factor 4E (eIF4E)," *Plant J.* 32:1067-1075 (2002),

25 which is hereby incorporated by reference in its entirety). Previous work has shown that at least one intron occurs in the eIF4E-687 locus because genomic PCR yielded a 1.8 kb amplicon, compared to a 493 bp cDNA product (Ruffel et al., "A Natural Recessive Resistance Gene Against Potato Virus Y in Pepper Corresponds to the Eukaryotic Initiation Factor 4E (eIF4E)," *Plant J.* 32:1067-

30 1075 (2002), which is hereby incorporated by reference in its entirety). Based on alignment of the primer sequences with genomic sequence of the locus, our data confirm that this amplicon would span three introns and have a total length of 1836 bp. The four intron sites were completely conserved based on homology of

pepper eIF4E-687 with *Arabidopsis* eIF4E. Exon length is also conserved between the two genes with the exception of exon 1, which is 20 bp longer in *Arabidopsis*.

5   **Example 31 – Resistance Alleles Have Distinct Amino Acid Substitutions**

[0159]   Amino acid alignments were used to compare eIF4E-687 alleles amplified from *Capsicum* germplasm known to be homozygous for $pvr1^+$, $pvr1$, $pvr1^1$ or $pvr1^2$. Three *C. chinense* PIs (152225, 159234, and 159236) previously shown to be allelic for $pvr1$ (Murphy et al., "Genetic Mapping of the pvr1 Locus
10   in *Capsicum spp.* and Evidence that Distinct Potyvirus Resistance Loci Control Responses that Differ at the Whole Plant and Cellular Levels," *Mol. Plant Microbe Interact.* 11:943-951 (1998), which is hereby incorporated by reference in its entirety) and three *C. annuum* bell pepper breeding lines known to carry $pvr1$ (3618, 4074, 5502) all carried identical sequence at the eIF4E-687 locus
15   (FIG. 2A), consistent with their known pedigrees of inter-specific introgression of resistance from *C. chinense* to *C. annuum*. The $pvr1$ allele carries four amino acid substitutions relative to susceptible *C. annuum* $pvr1^+$ genotypes (FIG. 2A).

[0160]   Bell pepper varieties 'Dempsey', 'Florida VR2', and 'Del Ray Bell' are known to be homozygous for $pvr1^2$. Their identical sequence at the
20   eIF4E-687 locus is also consistent with a commonly introgressed resistance gene. This allele had three point mutations that differed from susceptible *C. annuum* genotypes, all within the same region as the $pvr1$ point mutations but at different residues. This suggests the hypothesis that the $pvr1$ and $pvr1^2$ alleles originated independently in *C. chinense* and *C. annuum*, respectively. The amino acid
25   substitution L79R was not previously reported in the 'Florida VR2' eIF4E-687 sequence (Ruffel et al., "A Natural Recessive Resistance Gene Against Potato Virus Y in Pepper Corresponds to the Eukaryotic Initiation Factor 4E (eIF4E)," *Plant J.* 32:1067-1075 (2002), which is hereby incorporated by reference in its entirety), however, our results confirm this mutation is present in three
30   independent germplasm sources. The eIF4E-687 sequence from 'Yolo Y' ($pvr1^1$) shares both of its amino acid substitutions with $pvr1^2$. While the reported pedigrees do not identify a common ancestor for these two alleles (Cook, "A

Mutation for Resistance to Potato Virus Y in Pepper.," *Phytopathology* 51:550-552 (1961), which is hereby incorporated by reference in its entirety), their similar sequence is consistent with their shared resistance to PVY-0.

[0161]   Three of the four point mutations in the *pvr1* allele and all of the mutations in *pvr1*[1] and *pvr1*[2] cause non-conservative amino acid substitutions in the respective predicted proteins. Protein homology models based on mouse eIF4E predict conformational differences in the proteins as a result of the combination of mutations (FIG. 2B). It is also evident that in the three alleles, residues affected by these mutations localize to similar parts of the protein. Substitutions in *pvr1*, *pvr1*[1] and *pvr1*[2] are generally not located at highly-conserved residues involved in cap-binding or eIF4G binding, except in the case of the *pvr1*[2] D109R mutation. In contrast to earlier interpretation (Rodriguez et al., "The *Arabidopsis thaliana* cDNAs Coding for eIF4E and eIF(iso)4E are Not Functionally Equivalent for Yeast Complementation and are Differentially Expressed During Plant Development," *Plant J.* 13:465-473 (1998), which is hereby incorporated by reference in its entirety), this mutation does affect a highly conserved residue involved in stabilization of cap-binding (Marcotrigiano et al., "Cocrystal Structure of the Messenger RNA 5' Cap-Binding Protein (eIF4E) Bound to 7-Methyl-GDP," *Cell* 89:951-961 (1997), which is hereby incorporated by reference in its entirety). The *pvr1* allele has a G107R mutation two residues away from this position.

Example 32 – NIa/VPG Interaction with eIF4E-687 Is Not Correlated with TEV Resistance Phenotype

[0162]   The prevailing model indicates that interaction of eIF4E and NIa is required for potyviral infectivity (Leonard et al., "Complex Formation Between Potyvirus VPg and Translation Eukaryotic Initiation Factor 4E Correlates with Virus Infectivity," *J. Virol.* 74:7730-7737 (2000); Ruffel et al., "A Natural Recessive Resistance Gene Against Potato Virus Y in Pepper Corresponds to the Eukaryotic Initiation Factor 4E (eIF4E)," *Plant J.* 32:1067-1075 (2002); Schaad et al., "Strain-Specific Interaction of the Tobacco Etch Virus NIa Protein with the Translation Initiation Factor eIF4E in the Yeast Two-Hybrid System," *Virology* 273:300-306 (2000), which are hereby incorporated by reference in their entirety)

and predicts that conformational changes in eIF4E-687 gene products encoded by the potyvirus resistance alleles should abolish or impair NIa binding. In order to clarify how the mutations described above affect protein function and to examine whether the correlation between NIa binding and infectivity can be extended,

5   interaction of NIa with eIF4E-687 proteins using the yeast two-hybrid assay and interaction of VPg with eIF4E-687 proteins using the GST pull-down assay were examined. In the yeast two-hybrid system, five different eIF4E-687 alleles were analyzed for interaction with NIa from two different TEV strains, TEV-HAT and TEV-NW (FIG. 11A). Characteristic infectivity of TEV-HAT and TEV-NW on

10   homozygous $pvr1^+$, $pvr1$, $pvr1^1$ and $pvr1^2$ genotypes was confirmed by indirect ELISA. Results are depicted in FIG. 11B.

[0163]   Activity values from vector-only negative interaction controls and NIb positive interaction controls were consistent with those reported in previous studies using TEV NIa (Schaad et al., "Strain-Specific Interaction of the Tobacco

15   Etch Virus NIa Protein with the Translation Initiation Factor eIF4E in the Yeast Two-Hybrid System," *Virology* 273:300-306 (2000), which are hereby incorporated by reference in their entirety). The $pvr1^+$ alleles from TEV-susceptible genotypes RN and HAB interacted *in vitro* with NIa cloned from both viral isolates (FIG. 11A); RN and HAB developed severe infection when

20   inoculated with either TEV-HAT or TEV-NW (FIG. 11B). In contrast, fusion proteins from resistance alleles cloned from *Capsicum* genotypes 234 (*pvr1*), DEMP (*pvr1^2*), and YY (*pvr1^1*) failed to interact with NIa from either virus although they were expressed at levels similar to susceptible alleles. Both 234 and DEMP failed to support viral infection of TEV-HAT (FIG. 11B). In four cases,

25   however, where eIF4E-687/NIa interaction was interrupted, systemic viral infection was observed. YY (*pvr1^1*) is susceptible to both TEV-HAT and TEV-NW (FIG. 11B), even though eIF4E-687 isolated from YY failed to interact with NIa from either virus (FIG. 11A). Similarly, 234 (*pvr1*) and DEMP (*pvr1^2*) both support infection of TEV-NW even though there is no interaction *in vitro* between

30   their respective eIF4E-687 alleles and TEV-NW NIa.

[0164]   These data were confirmed and extended by a second assay of *in vitro* interaction shown in FIG. 12. Results confirmed that interaction was only observed between eIF4E-RN and TEV-HAT and TEV-NW VPg. No interaction

was observed between the resistant alleles and either TEV-HAT or TEV-NW (FIG. 12).

[0165]   These data uniformly supported the prediction that the translational products of *pvr1*, *pvr1*[1] and *pvr1*[2] alleles would show altered function with respect

5 to NIa/VPg binding. Interruption of the interaction, however, was not uniformly correlated with failure to infect *in vivo*. Strong NIa/VPg/eIF4E-687 interaction in highly susceptible *pvr1*[+] hosts may promote potyviral infectivity, but to the extent that *in vivo* conditions can be extrapolated from *in vitro* assays, disruption of this interaction does not necessarily abolish infection.

10

Example 33 – Cap-Binding Activity of eIF4E-687 Is Not Correlated with TEV Resistance Phenotype

[0166]   In order to determine whether eIF4E-687 proteins were functional with respect to their cellular role of cap-binding, *pvr1*[+], *pvr1*, *pvr1*[1] and *pvr1*[2]

15 alleles were expressed in *E. coli* and resulting proteins were assayed for binding-activity on m[7]-GTP cap-analog columns. All of the eIF4E-687 proteins were stably expressed in *E.coli* (FIG. 13A). The *pvr1*[+] eIF4E-687 allele from the susceptible host RN showed strong cap-binding activity, as did recombinant DEMP (*pvr1*[2]) and YY (*pvr1*[1]) eIF4E-687 proteins (FIG. 13) indicating that the

20 D109R mutation specific to *pvr1*[2] did not abolish cap-binding. In contrast, the 234 (*pvr1*) product failed to bind the column. It is unclear whether this failure to bind was a result of the G107R mutation, or due to other disruptions in the protein. Plants homozygous for *pvr1* are phenotypically normal in every respect, suggesting that the cap-binding activity of eIF4E-687 may not be essential for host

25 viability. Furthermore, the observation that point mutations in two resistance alleles failed to disrupt cap-binding while disruption was observed in the third case is consistent with the hypothesis that cap-binding or its disruption may not be essential for the resistant response. It remains unclear whether this attribute of the *pvr1* allele will in some way account for or contribute to its relatively broad

30 spectrum of resistance.

[0167]   These studies confirm that eIF4E-687 is the host factor responsible for recessive potyvirus resistance attributed to *pvr1* and alleles formerly known as *pvr2*[1] and *pvr2*[2]. A genetic complementation test confirmed that all three alleles

occur at the same locus, requiring revision of the locus designation. Hybridization and sequence-based markers for the eIF4E-687 gene co-segregated completely with the *pvr1* resistance phenotype in two independent populations. Furthermore, the wild type allele showed functional complemention when transiently expressed

5 via agroinfiltration in a resistant genotype restoring susceptibility to TEV-HAT. Each resistant allele carried signature point mutations localized to similar regions of the gene. These data, together with a recent study that demonstrated that transient expression of wild-type eIF4E-687 in *pvr1*[1] and *pvr1*[2] genotypes restored susceptibility to PVY (Ruffel et al., "A Natural Recessive Resistance Gene

10 Against Potato Virus Y in Pepper Corresponds to the Eukaryotic Initiation Factor 4E (eIF4E)," *Plant J.* 32:1067-1075 (2002), which is hereby incorporated by reference in its entirety), clearly indicate that mutations in this gene can result in broad spectrum potyvirus resistance. It is now clear from studies in *Arabidopsis*, lettuce and *Capsicum* and that cap-binding proteins play an important, if

15 undefined, role in potyvirus infection.

[0168]   VPg has been proposed to act as a viral determinant of infectivity in these resistance systems based on the identity of these resistance genes (Ruffel et al., "A Natural Recessive Resistance Gene Against Potato Virus Y in Pepper Corresponds to the Eukaryotic Initiation Factor 4E (eIF4E)," *Plant J.* 32:1067-

20 1075 (2002); Nicaise et al., "The Eukaryotic Translation Initiation Factor 4E Controls Lettuce Susceptiblity to the Potyvirus Lettuce Mosaic Virus," *Plant Phys.* 132:1272-1282 (2003), which are hereby incorporated by reference in their entirety). Avirulence determinants have not yet been established for race-specific resistance conferred by the *Capsicum* eIF4E-687 mutants. There are, however,

25 amino acid differences within the VPg region of TEV strains virulent (TEV-NW) and avirulent (TEV-HAT) on *pvr1* and *pvr1*[2] and differences within the VPg region of PVY strains virulent and avirulent on *pvr1*[2] (Ruffel et al., "A Natural Recessive Resistance Gene Against Potato Virus Y in Pepper Corresponds to the Eukaryotic Initiation Factor 4E (eIF4E)," *Plant J.* 32:1067-1075 (2002), which is

30 hereby incorporated by reference in its entirety). Additional studies focusing on infectivity of chimeric infectious clones constructed between TEV-NW and TEV-HAT in *Capsicum* are currently underway to address this question.

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[0169]   Based upon results from a previous study which noted that a
mutation in TuMV NIa (D77N) that abolished *Arabidopsis* eIF(iso)4E interaction
also abolished infection in another host plant, *Brassica perividis* (Leonard et al.,
"Complex Formation Between Potyvirus VPg and Translation Eukaryotic

5   Initiation Factor 4E Correlates with Virus Infectivity," *J. Virol.* 74:7730-7737
(2000), which is hereby incorporated by reference in its entirety), it was proposed
that the outcome of the interaction of eIF4E-687 and NIa would determine viral
infectivity. It was not apparent from that study, however, whether this mutation in
a highly conserved NIa residue acted only to interrupt interaction with eIF(iso)4E

10   or caused some other instability in the NIa protein. Furthermore, the correlation
was not tested directly by inoculating *Arabidopsis* with the mutant virus. In
addition to the demonstrated interaction between TuMV NIa and translation
factors eIF(iso)4E and eIF4E from the host species *Arabidopsis*, interaction was
also observed in that study between TuMV NIa and eIF4E from wheat, a nonhost

15   for the virus. This result is inconsistent with hypotheses that posit that the
interaction between eIF4E and NIa is causal for infectivity, although it is possible
that the correlation holds true only for eIF(iso)4E and not eIF4E.

[0170]   Observations show that there is strong interaction of *pvr1$^+$* eIF4E-
687 proteins from *C. annuum* and *C. chinense* with NIa from TEV-HAT and

20   TEV-NW, both of which cause severe infection. Absence of interaction was
observed between the avirulent TEV-HAT NIa and resistant *pvr1* and *pvr1^2*
eIF4E-687 alleles. However, also observed was a lack of interaction in four
NIa/eIF4E assays that represent cases where the outcome of infection is
susceptibility (TEV-HAT NIa/*pvr1^1*; TEV-NW NIa/*pvr1*; TEV-NW NIa/*pvr1^1*;

25   TEV-NW NIa/*pvr1^2*). This result is clearly inconsistent with the hypothesis that
this interaction predicts or determines the ability of a viral isolate to infect.

[0171]   Results from other studies are consistent with the conclusion that
eIF4E/NIa interaction is important but its outcome does not necessarily determine
resistance. NIa from TEV-HAT is the avirulence determinant for recessive

30   resistance to systemic movement in tobacco cultivar V20 (Schaad et al., "VPg of
Tobacco Etch Potyvirus is a Host Genotype-Specific Determinant for Long-
Distance Movement," *J. Virol.* 71:8624-8631 (1997), which is hereby
incorporated by reference in its entirety). In an effort to identify host factors

interacting with TEV-HAT NIa, a yeast two-hybrid screen identified tomato eIF4E from TEV susceptible variety VF36 as a strong interactor (Schaad et al., "Strain-Specific Interaction of the Tobacco Etch Virus NIa Protein with the Translation Initiation Factor eIF4E in the Yeast Two-Hybrid System," *Virology* 273:300-306 (2000), which is hereby incorporated by reference in its entirety). However, this eIF4E protein failed to interact with TEV-Oxnard NIa, even though VF36 is also susceptible to that virus. When eIF4E sequence was analyzed between the resistant tobacco cultivar V20 and two susceptible tobacco varieties, there was no difference, indicating that the gene was not involved in V20 resistance. Thus, NIa in this system is an avirulence determinant for a resistance mechanism that apparently does not involve eIF4E. Yeast two-hybrid assays confirmed that the tobacco eIF4E protein interacted with NIa from TEV-HAT, even though V20 blocked systemic movement of the virus; however in the same assay, eIF4E failed to interact with NIa from TEV-Oxnard, even though TEV-Oxnard can infect all three varieties. Experiments using chimeric NIa proteins carrying the minimal avirulence determinant suggested that the interaction determinants were genetically distinct from the avirulence determinant. Overexpression of eIF4E within the TEV genome in the same study appeared to have a stimulatory effect on replication, although there is no evidence that this stimulation involved interaction between VPg-Pro and eIF4E.

[0172]   One important observation from the present study is that in one of the three alleles, the ability to bind capped mRNA is interrupted. This clearly demonstrates that impaired cap-binding ability is not essential for potyvirus resistance, however the relevance of this observation, if any, to the distinctive phenotypic features of this allele, e.g., increased resistance spectrum, remains to be determined. Premature stop codons and insertions into *Arabidopsis* eIF(iso)4E almost certainly interrupt both VPg and cap-binding and led to broad-spectrum potyvirus resistance in *Arabidopsis* (Duprat et al., "The Arabidopsis Eukaryotic Initiation Factor (iso)4E is Dispensable for Plant Growth but Required for Susceptibility to Potyviruses," *Plant J.* 32:927-934 (2002); Lellis et al., "Loss-of-Susceptibility Mutants of *Arabidopsis thaliana* Reveal an Essential Role for eIF(iso)4E During Potyvirus Infection," *Curr. Biol.* 12:1046-1051 (2002), which are hereby incorporated by reference in their entirety). A second implication of

this result is that impaired cap-binding of eIF4E-687 has no apparent phenotypic consequences in *pvr1* plants, consistent with functional redundancy in *Capsicum*. Specifically, it is possible that eIF4E-537, eIF(iso)4E, or other molecules may function in place of mutant eIF4E-687 in *pvr1* genotypes. Likewise, no

5   phenotypic defects were found in potyvirus resistant *Arabidopsis* eIF(iso)4E mutants, suggesting that redundancy for this function may be universal in plants. Although it is possible that *Capsicum* eIF4E-687 has cellular functions other than cap-binding that are as yet unknown, results imply that eIF4E-687 is a homolog whose function may be dispensable for the host, but essential for the virus. This

10 homolog, therefore, defines a target for transgenic gene-silencing techniques to generate viable crop plants with stable engineered resistance to potyviruses.

[0173]   The pattern of amino acid substitutions observed among *pvr1*, *pvr1*[1] and *pvr1*[2] alleles depicted in FIG. 12 suggest testable hypotheses regarding the molecular basis for strain-specificity, the first such system to be defined. The

15 *pvr1* G107R mutation and *pvr1*[2] D109R mutations within the cap-binding slot may have similar effects on host-potyvirus interaction and result in resistance to TEV-HAT and PVY-1 common to both alleles. Conformational differences resulting from the two unique mutations, each in the region flanking the cap-binding slot allele (P66T and K71R in *pvr1*, V67E and L79R in both *pvr1*[1] and

20 *pvr1*[2]) may have similar consequences for resistance to PVY-0. Finally, the combined effect in *pvr1* of the T51A mutation together with the other three mutations may serve to extend the resistance spectrum to include PepMoV and PVY-1, 2. It is unclear why the *pvr1*[2] K71R mutation was not previously reported, but confirmation from three independent cultivars that carry this allele,

25 including 'Dempsey', the focus of *pvr1*[2] mechanistic studies (Deom et al., "Resistance to Tobacco Etch Virus in *Capsicum annuum*: Inhibition of Virus RNA Accumulation," *Mol. Plant Microbe Interact.* 10:917-921 (1997), which is hereby incorporated by reference in its entirety), supports this finding.

[0174]   Models regarding the molecular basis of resistance at the *pvr1*

30 locus must account for two different resistance mechanisms (replication vs. cell-to-cell movement) attributed to this locus, and for the observation that putative structural differences in eIF4E-687 correlate with differences in strain-specificity. The simplest model would suggest that these differences would be coupled with

virus strain-specific structural differences in the VPg molecule, the eIF4E binding domain of NIa, to produce an interaction phenotype that determines resistance or susceptibility. This model appears to hold in the case of $pvr1^{+}$ genotypes and in the case of $pvr1$ and $pvr1^{2}$ resistance to TEV-HAT. However, in four cases the

5 lack of eIF4E-687 interaction with VPg did not correlate with resistance to the virus. Under these circumstances, other factors must compensate to promote systemic infection. Within plant cells, interaction of eIF4E-687 and VPg would likely be influenced by conformational changes brought on by interaction with other host and viral proteins and RNA. Therefore, there may be alternate ways to

10 stabilize association of eIF4E-687 with VPg or to substitute for the function the interaction provides. In the case of $pvr1$ and $pvr2^{2}$ infection with TEV-NW and $pvr1^{1}$ infection with TEV-HAT, symptom development typically begins a few days later and proceeds at a slightly slower rate than that seen in $pvr1^{+}$ genotypes. This might indicate a modified mode of infection by the virus when faced with

15 eIF4E-687 proteins that fail to interact with VPg.

[0175]   Interaction of VPg with eIF4E has been proposed to confer a translational advantage to the virus (Lellis et al., "Loss-of-Susceptibility Mutants of *Arabidopsis thaliana* Reveal an Essential Role for eIF(iso)4E During Potyvirus Infection," *Curr. Biol.* 12:1046-1051 (2002), which is hereby incorporated by

20 reference in its entirety), however there is evidence from both potyvirus and poliovirus that both eIF4E and VPg are dispensable for cap-independent translation (Gallie, "Cap-Independent Translation Conferred by the 5' Leader of Tobacco Etch Virus is Eukaryotic Initiation Factor 4G Dependent," *J. Virol.* 75:12141-12152 (2001); Nomoto et al., "The 5'-Terminal Structures of

25 Poliovirion RNA and Poliovirus mRNA Differ Only in the Genome-Linked Protein VPg," *Proc. Natl. Acad. Sci. USA* 74:5345-5349 (1977); Sachs et al., "Starting at the Beginning, Middle, and End: Translation Initiation in Eukaryotes," *Cell* 89:831-838 (1997), which are hereby incorporated by reference in their entirety). In many RNA virus-host systems, interaction between cellular

30 translation machinery, viral proteins and RNA appears to be important in bringing 5' and 3' regulatory elements together for RNA replication (Herold et al., "Poliovirus RNA Replication Requires Genome Circularization Through a Protein-Protein Bridge," *Mol. Cell* 7:581-591 (2001); Lai, "Cellular Factors in the

Transcription and Replication of Viral RNA Genomes: A Parallel to DNA-Dependent RNA Transcription," *Virology* 244:1-12 (1998), which are hereby incorporated by reference in their entirety). Interaction of these regulatory regions are proposed to favor viral replication for a number of reasons, including efficient

5   recruiting of the RNA polymerase to the replication start site, ensuring that only intact viral templates are replicated, and coordinating RNA replication with translation (Herold et al., "Poliovirus RNA Replication Requires Genome Circularization Through a Protein-Protein Bridge," *Mol. Cell* 7:581-591 (2001), which is hereby incorporated by reference in its entirety). TEV is known to have

10   RNA secondary structures at the 3' end of its genome that are required in *cis* for RNA replication (Haldeman-Cahill et al., "Secondary Structures in the Capsid Protein Coding Sequence and 3' Nontranslated Region Involved in Amplification of the Tobacco Etch Virus Genome," *J. Virol.* 72:4072-4079 (1998); and Mahajan et al., "Roles of the Sequence Encoding Tobacco Etch Virus Capsid Protein in

15   Genome Amplification: Requirements for the Translation Process and a Cis-Active Element," *J. Virol.* 70:4370-4379 (1996), which are hereby incorporated by reference in their entirety). With VPg attached to the 5' end of the potyviral genome, interaction with eIF4E-687 could create a bridge to the 3' poly-A tail through interaction with eIF4G and poly-A binding protein, thus allowing

20   regulation of RNA synthesis. Mutations in eIF4E-687 that interrupt this association could explain how *pvr1* and *pvr1²* genotypes block replication of TEV-HAT.

[0176]   Isolated protoplasts from plants homozygous for *pvr1¹* accumulate PVY-0 to susceptible levels (Arroyo et al., "Impaired Cell-to-Cell Movement of

25   Potato Virus Y in Pepper Plants Carrying the Y-A (pr2-1) Resistance Gene," *Mol. Plant Microbe Interact.* 9:314-318 (1996), which is hereby incorporated by reference in its entirety), suggesting that this allele does not share the same functional defect as described above for *pvr1* and *pvr1²*. The interaction between NIa from PVY pathotypes and this eIF4E allelic series has not been examined *in*

30   *vitro*, but binding properties in the *pvr1¹* mutant may be altered in such a way to affect VPg availability and/or function. One possibility is that eIF4E-687 may be recruited by VPg to the origin of virion assembly, shown to be near the 5'

terminus of the viral genome (Wu et al., "Evidence That Assembly of a Potyvirus Begins Near the 5' Terminus of the Viral RNA," *J. Gen. Virol.* 79:1525-1529 (1998), which is hereby incorporated by reference in its entirety), and somehow affect the assembly process. Association of the viral genome with coat protein is essential for potyvirus movement (Revers et al., "New Advances in Understanding the Molecular Biology of Plant/Potyvirus Interactions," *Mol. Plant Microbe Interact.* 12:367-376 (1999), which is hereby incorporated by reference in its entirety) and disruption of this association could potentially result in blocked cell-to-cell movement.

[0177]   Even though the precise mechanisms through which cap-binding factors promote potyvirus infection have yet to be resolved, it is now clear that both eIF4E and eIF(iso)4E are strong candidates for a number of naturally occurring recessive potyvirus resistance genes. Based on mapping data in tomato and pepper, respectively, *pot-1* and *pvr5* are likely to be eIF4E-687 homologs (Parella et al., "Recessive Resistance Genes Against Potyviruses are Localized in Colinear Genomic Regions of the Tomato (Lysopersicon spp.) and Pepper (*Capsicum spp.*) Genomes," *Theor. Appl. Genet.* 105:855-861 (2002), which is hereby incorporated by reference in its entirety). Results presented here indicate that *pvr6*, which is reported to confer resistance to PVMV when combined with *pvr1²* (Caranta et al., "A Complementation of Two Genes Originating from Susceptible *Capsicum annuum* Lines Confers a New and Complete Resistance to Pepper Veinal Mottle Virus," *Phytopathology* 86:739-743 (1996), which is hereby incorporated by reference in its entirety), is likely to be eIF(iso)4E. The combined effects of mutations in two cap-binding isomers may also explain the epistatic effect of the recessive *bc-u* gene on recessive bean common mosaic virus (BCMV) resistance genes *bc-1*, *bc-2* and *bc-3* (Drijfhout, "Genetic Interaction Between *Phaseolus Vulgaris* and Bean Common Mosaic Virus with Implications for Strain Identification and Breeding for Resistance," *Agricultural Research Reports* 872 (1978), which is hereby incorporated by reference in its entirety). In other recessive potyvirus-host systems, candidate gene analysis will reveal the universality of eIF4E and eIF(iso)4E mutants (Keller et al., "Potyvirus Genome-Linked Protein (VPg) Determines Pea Seed-Borne Mosaic Virus Pathotype-Specific Virulence in *Pisum sativum*," *Mol. Plant Microbe Interact.* 11:124-130

(1998); Nicolas et al., "Variations in the VPg Protein Allow a Potyvirus to Overcome va Gene Resistance in Tobacco," *Virology* 237:452-459 (1997), which are hereby incorporated by reference in their entirety).

[0178]   The apparently dispensable eIF4E homolog, eIF4E-687, may define an opportunity to engineer extreme cellular-level resistance by targeted silencing in transgenic plants (Azevedo et al., "The RAR1 Interactor SGT1, an Essential Component of R Gene-Triggered Disease Resistance," *Science* 295:2073-2076 (2002), which is hereby incorporated by reference in its entirety). As more is learned that links functional shifts in resistance mechanism and specificity to defined changes in interacting host and pathogen molecules, the *pvr1* system offers a unique opportunity to extend our understanding of the role host gene products play in viral pathogenesis, and the molecular determinants of host-pathogen specificity. Further, this study confirms eIF4E and eIF(iso)4E as viable candidates for a plethora of naturally occurring virus resistance genes for which molecular markers may be very useful in agriculture and may open the prospect for superior transgenic strategies to engineer highly durable resistance to control an extremely broad set of plant viruses.

Example 34 – Transgenic Tomato Plants Containing Heterologous Sense eIF4E Genes: Plasmid Constructs

[0179]   The plasmids for tomato transformation were derived from pBI121. The full length ORFs of eIF4E genes were PCR amplified from cDNA of pepper eIF4E ('PI159234' and 'RNaky'), and tomato eIF4E (Rio Grande). GUS gene of pBI121 was replaced by the PCR amplified pepper and tomato eIF4E genes. The resulting plasmids constructs were designated pBI::eIF4E-234S, pBI::eIF4E-RNS, and pBI::eIF4E-LeS. In addition to the constructs, pBI121 with and without GUS gene were also used as negative controls.

Example 35 – Transgenic Tomato Plants Containing Heterologous Sense eIF4E Genes: Tomato Transformation

[0180]   Microtom tissues were transformed and regenerated into whole plants as described earlier. About 46 transgenic plants were obtained from the constructs and eIF4E gene expression was tested by Northern blot analysis.

Example 36 – **Transgenic Tomato Plants Containing Heterologous Sense eIF4E Genes: Virus Resistance Test**

[0181]   Seventeen transgenic plants showing either high or low eIF4E gene expression were tested for TEV virus resistance (see Table 3).

Table 3 — Resistance to TEV Infection of Tomato Transgenic Plants Containing Sense eIF4E Genes of Pepper and Tomato

Constructs	Tested line	eIF4E expression	Plant phenotype
pBI::eIF4E-234	234-S2	H	Resistance segregates
	234-S6	H	Resistance segregates
	234-S7	L	All susceptible
	234-S9	H	Resistance segregates
	234-S10	H	Resistance segregates
	234-S13	H	Resistance segregates
pBI::eIF4E-RN	RN-S1	H	All susceptible
	RN-S2	H	All susceptible
	RN-S3	L	All susceptible
	RN-S4	H	All susceptible
	RN-S8	H	All susceptible
	RN-S11	H	All susceptible
pBI::eIF4E-TAB	Le-S2	H	All susceptible
	Le-S4	H	All susceptible
	Le-S8	H	All susceptible
	Le-S9	H	All susceptible

[0182]   Tomato plants, at 4–6 leaf stage, were inoculated with sap extract from TEV infected tobacco leaves (5 ml of 50 mM sodium phosphate buffer per g of tissue) and scored for symptoms 14 days after inoculation. TEV coat protein accumulation in inoculated plants was tested by a dot blotting analysis after 21 days after inoculation. Dot blotting analysis was carried out as follows: A leaf disc (microtube cap size) was collected from each plant and ground in 50 volume of extraction buffer (1 X TBS containing 50 mM diethyldithiocarbamate). The sample was centrifuged at 10,000 g for 2 min and diluted 100 times. About 40 µl of diluted extract was spotted on nitrocellulose membrane. The membrane was blocked with 5% BSA in 1X TBS and hybridized with TEV coat protein antibody and signal was developed using ECL detecting system according to manufacturer's instruction.

Example 37 – Transgenic Tomato Plants Containing Heterologous Sense eIF4E Genes: Results and Analysis

[0183]   Untransformed plants, pBI121 transgenic plants, and pBI121 empty vector transgenic plants showed TEV symptoms and TEV coat protein accumulation (Fig. 14). All the progenies of tomato transgenic plants of pBI::eIF4E-RNS and pBI::eIF4E-LeS, which contain functional eIF4E gene, showed susceptible phenotype to TEV virus regardless of eIF4E expression level. The progenies of tomato transgenic plants pBI::eIF4E-234-S6, S9, S10, and S13, which contain mutated eIF4E gene, showed resistant and susceptible phenotype segregation, where resistant phenotype was dominant. Resistant plants showed complete resistance, no TEV symptoms and no TEV coat protein accumulation (Fig. 14). However, resistant phenotype was observed only when the transgenic plants accumulate high level of eIF4E transcript (Table 3). These results indicate that TEV resistant plants can be engineered by overexpressing eIF4E-234 gene.

[0184]   Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.